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Genetics

Generation of a high-titer retroviral vector capable of expressing high levels of the human β-globin gene

(hemoglobinopathies/gene therapy/locus control region)

MICHEL SADELAIN*†, C. H. JASON WANG*, MICHAEL ANTONIOU‡, FRANK GROSVELD‡§, AND RICHARD C. MULLIGAN*¶

*Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Nine Cambridge Center, Cambridge, MA 02142-1479; and ‡Laboratory for Gene Structure and Expression, Medical Research Council National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7, United Kingdom

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ABSTRACT **Retrovirus-mediated gene transfer into hematopoietic cells may provide a means of treating both inherited and acquired diseases involving hematopoietic cells. Implementation of this approach for disorders resulting from mutations affecting the β-globin gene (e.g., β-thalassemia and sickle cell anemia), however, has been hampered by the inability to generate recombinant viruses able to efficiently and faithfully transmit the necessary sequences for appropriate gene expression. We have addressed this problem by carefully examining the interactions between retroviral and β-globin gene sequences which affect vector transmission, stability, and expression. First, we examined the transmission properties of a large number of different recombinant proviral genomes which vary both in the precise nature of vector, β-globin structural gene, and locus control region (LCR) core sequences incorporated and in the placement and orientation of those sequences. Through this analysis, we identified one specific vector, termed MβδL, which carries both the human β-globin gene and core elements HS2, HS3, and HS4 from the LCR and faithfully transmits recombinant proviral sequences to cells with titers greater than 10^6 per ml. Populations of murine erythroleukemia (MEL) cells transduced by this virus expressed levels of human β-globin transcript which, on a per gene copy basis, were 78% of the levels detected in an MEL-derived cell line, Hu11, which carries human chromosome 11, the site of the β-globin locus. Analysis of individual transduced MEL cell clones, however, indicated that, while expression was detected in every clone tested (n = 17), the levels of human β-globin treatment varied between 4% and 146% of the levels in Hu11. This clonal variation in expression levels suggests that small β-globin LCR sequences may not provide for as strict chromosomal position-independent expression of β-globin as previously suspected, at least in the context of retrovirus-mediated gene transfer.**

The successful treatment of β-globin disorders by gene therapy will likely require the efficient and stable introduction of a functional β-globin gene into self-renewing hematopoietic stem cells. While we and others had established that retroviral vectors encoding the human β-globin structural gene could direct the erythroid-specific synthesis of β-globin in mice engrafted with genetically modified bone marrow cells (1–4), these studies suffered from two important limitations. First, the retroviral titers generally obtained with those vectors made it difficult to generate long-term bone marrow chimeras engrafted with efficiently transduced cells. A second problem was the variable and generally low level of human β-globin expression *in vivo* observed in the reconstituted animals.

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The prospects for increasing the level of β-globin expression obtainable in retroviral vectors were dramatically improved by the recent finding that DNA sequences located considerable distances from the human β-globin structural gene on chromosome 11, termed locus control region (LCR) sequences (5, 6), play a critical role in the transcriptional control of genes within the β-globin-like gene cluster (reviewed in refs. 7–9). Specifically, transgenic experiments from a number of laboratories have indicated that the linkage of LCR sequences to the human β-globin structural gene dramatically increases the level of expression of β-globin observed in erythroid cells (10–13). Some studies further suggested that LCR sequences may differ from classical transcriptional enhancer sequences in that they provide for the chromosomal position-independent expression of linked genes (10–16). Smaller fragments, more suitable for insertion into vectors, have recently been defined, which have been reported to retain partial LCR activity (17–29). Recent efforts by several groups to incorporate LCR subfragments into β-globin retroviral vectors, however, have resulted in vector rearrangements (ref. 30 and our observations), poor titers (31), or very low expression of the β-globin gene (32).

We report here a systematic study of the β-globin gene, LCR core sites, and retroviral sequences that control vector transmission. This analysis has led to the generation of a high-titer, genomically stable retroviral vector bearing the human β-globin gene and the LCR core sites HS2, HS3, and HS4. This vector confers elevated and erythroid-specific expression of the β-globin gene. Expression in different clones of transduced murine erythroleukemia (MEL) cells is variable, however, raising questions about the ability of small LCR sequences to confer position-independent gene expression, at least in the context of a retroviral vector.

MATERIALS AND METHODS

Generation of Vectors and Packaging Cell Lines. The retroviral vectors pSG and pMFG are described elsewhere (33, 34). The β-globin gene was subcloned from pSVX-Neo (1). The β-globin coding sequence (βCS) consists of a 444-bp *Nco* I-linker-containing β-globin sequence from the translational start to the stop codon derived from the cDNA. The HS2, HS3, and HS4 fragments (see text and refs. 19–22 and 27–29) were, respectively, given *Hin*dIII, *Eco*RI, and *Mlu* I linkers and subcloned in all possible orientations in a *Sac* II–*Hin*dIII–

Abbreviations: LCR, locus control region; LTR, long terminal repeat; MEL, murine erythroleukemia; MLV, murine leukemia virus.

†Present address: Department of Human Genetics, Memorial Sloan–Kettering Cancer Center, Box 182, 1275 York Avenue, New York, NY 10021.
§Present address: Department of Cell Biology, Erasmus University, Postbus 1738, 3000 Dr Rotterdam, The Netherlands.
¶To whom reprint requests should be addressed.

*Eco*RI–*Mlu* I–*Sac* II polylinker. The vectors described herein, lacking any selectable marker, were introduced by calcium phosphate cotransfection with pSV2Neo (35) into the ψ-CRE packaging cell line (36). After 10 days of selection in G418 (Geneticin, Sigma) at 1 mg/ml, the G418-resistant colonies (>50 per transfection) were pooled by trypsinization and grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, penicillin, and streptomycin without G418.

Fibroblast Infection and Viral Transmission Assay. Five milliliters of supernatant harvested from pooled producer cells was applied onto 10^6 NIH 3T3 fibroblasts. Polybrene (Sigma) was added at 8 μg/ml. After 4 hr, the conditioned medium was removed. Three days later, genomic DNA was extracted from the confluent NIH 3T3 fibroblasts. For Southern analysis, 10-μg samples of genomic DNA from the producer and the corresponding 3T3 target cells were digested overnight with the restriction enzyme *Nhe* I [which cuts in both long terminal repeats (LTRs)] and run side by side on a 1% agarose gel. [^{32}P]dCTP-labeled β-globin probes were generated by using the human *Nco* I–*Eco*RI genomic template. Band intensity was quantified by using a phosphorimager (Fuji Bio-Imaging). Vector signal intensity was normalized to that of the endogenous band. Very even loading in Figs. 1–3 allows for direct reading of the gels as shown.

MEL Cell Infection and Screening. C88 MEL cells were grown in RPMI medium/10% fetal bovine serum/penicillin/streptomycin. Infection was performed by a 48-hr cocultivation of 10^5 MEL cells, treated for the previous 18 hr with tunicamycin (Sigma) at 0.2 μg/ml, on the ecotropic producer in the presence of Polybrene at 4 μg/ml. After cocultivation, MEL cells were subcloned onto 96-well plates at 0.2 cell per well. Single clones were then expanded and genomic DNA was prepared. Infected clones were screened by PCR using the primers GCAAGAAAGTGCTCGGTG in exon 2 and TCTGATAGGCAGCCTGCA in exon 3. The DNA of positive clones was further analyzed by Southern blotting for copy number and integration site.

MEL Cell Induction and Globin mRNA Quantification. After 4 days in 2% (vol/vol) dimethyl sulfoxide, RNA was extracted from each MEL cell clone by using the lithium chloride/urea procedure (37). This induction-extraction procedure was repeated at least once. RNA was quantified by RNase protection (38) using probes described in ref. 39 for human β-globin and a 180-nucleotide *Pst* I–*Bam*HI genomic fragment (40) for mouse α-globin. One to 2 μg of RNA per extract was incubated with both admixed probes, which, after RNase digestion, were electrophoresed on a 6% polyacrylamide/urea gel. Signal intensity for each band (the α-globin serving as internal control for both induction and loading onto the gel) was determined by using the phosphorimager.

RESULTS

Features of Retroviral Vector Design Which Affect Transmission of the Human β-Globin Structural Gene. To assess the titer and genomic stability of different retroviral vectors bearing the human β-globin gene, we first developed a direct assay to characterize and quantitate vector transmission. This assay is based on a quantitative Southern blot analysis of vector copy number in both transfected packaging cells and infected 3T3 fibroblasts. Supernatant is harvested from a pool of stable virus-producing cell clones and used to infect the target fibroblasts under defined conditions (see *Materials and Methods*). Southern blot analysis of vector DNA copy number in the polyclonal producer and target cells allows for a direct evaluation of average transmission for any retroviral construct. This is achieved by direct comparison of signal intensity in both genomic DNA extracts run side by side and therefore does not rely on indirect readouts dependent on gene expression (such as G418 resistance). This method provides not only for a direct measurement of gene transfer but also for the detection of rearrangements of any given construction. As shown in Fig. 1, transmission of a minimal 1.8-kb β-globin gene and promoter (−129 to +1650) cloned in reverse orientation in the pSG vector (33) was not detectable in this assay (lanes A). Transmission of the larger 2.8-kb *Sph* I–*Pst* I (−615 to +2163) globin fragment was also not detectable (data not shown). In contrast, the β-globin coding sequence alone transmitted very well, showing comparable signals in the packaging and target cell DNAs (lanes B). The high titer of the latter vector, however, was greatly reduced by inclusion of the core sites HS2, HS3, and HS4 of the LCR (see below), which were introduced in the vector upstream of the globin sequence in either orientation (lanes C and D).

To determine which β-globin genomic sequences were responsible for the dramatic reduction in titer, we constructed a set of recombinant vectors bearing systematic deletions of untranslated regions of the gene. As shown in Fig. 2, deletion of the 5′ untranslated region and intron 1, either alone or in combination, failed to increase viral titers (lanes A–D). Deletion of intron 2 (lanes E) increased transmission to levels similar to the coding sequence vector (lanes I), yet further combined deletions did not increase titer any more (lanes F–H). Because β-globin DNA templates lacking intron 2 direct poor β-globin expression (refs. 24 and 41–44 and data not shown), we examined transmission of two subintronic deletions (from +518 to +1288 and +679 to +953). Both yielded similar titers, which were still 10-fold down from the intronless gene (shown for the first deletion, lanes J, Fig. 2). Therefore, intron 2 alone accounts for reduced titers of β-globin gene, and deletions within intron 2, while increasing titer, still transmit less well than intronless constructions.

Recombinant Human β-Globin Genes Yield Higher Titers in the pMFG Vector. Since decreased viral transmission may result from a negative interaction between β-globin and retroviral sequences, which is only partially alleviated by intron 2 subdeletions, we compared transmission of β-globin sequences in different vectors. Modifications of the length of gag sequence, replacement of the Moloney murine leukemia virus (MLV) packaging signal by that of Ha-Ras MLV, and mutation of the splice donor site present in pSG failed to increase vector transmission (data not shown). Subcloning recombinant β-globin fragments into the pMFG vector (34), however, significantly increased transmission (Fig. 3). Lanes A and D show transmission of the full β-globin fragment; lanes B and E, the genomic sequence bearing the intron 2 deletion shown

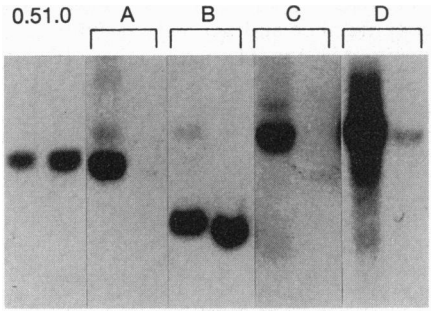

FIG. 1. Comparison of vector transmission by Southern blot analysis of vector copy number in the packaging cell (left lane) and target cell (right lane) genomic DNA. In lanes A, 1.8-kb β-globin gene and promoter; in lanes B, βCS; in lanes C and D, βCS and the three core elements HS2, HS3, and HS4 in sense and antisense orientation, respectively. See text for sequence description. Genomic DNA was digested with *Nhe* I and blots were probed with a β-globin probe. Control lanes show signal intensity for one proviral copy per cell (1.0) and one copy per two cells (0.5). Even loading (data not shown) allows for directly comparing signal intensity between lanes.

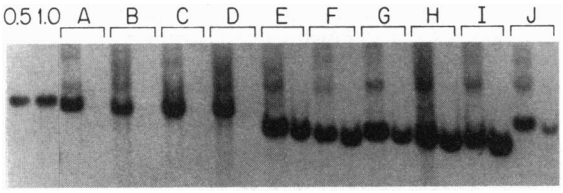

FIG. 2. Effect of β-globin gene untranslated sequences on vector transmission. Southern blot analysis of packaging cell (ψ-CRE, left lane) and target cell (NIH 3T3, right lane) genomic DNA. Lanes: A, 1.8-kb β-globin gene and promoter; B, 5′ untranslated region deletion; C, intron 1 deletion; D, B and C combined deletions; E, intron 2 deletion; F, B and E combined deletions; G, C and E combined deletions; H, B, C, and E combined deletions; I, β-globin coding sequence only; J, 770-bp intron 2 deletion. See Fig. 1 legend and text for sequences and methods.

in lanes J of Fig. 2; and lanes C and F, the globin coding sequence, in the vectors pSG and pMFG, respectively. Comparison between lanes B and E shows a gain in titer of about 5-fold when the pMFG vector is used, showing that altering vector sequences can affect transmission.

The Core Sites of HS2, HS3, and HS4 Can Be Incorporated into a High-Titer Genomically Stable Retroviral Vector. Previous studies of LCR sequences had indicated that a "minilocus" approximately 20 kb in size, comprising sites HS1, HS2, HS3, and HS4, possessed close to complete LCR activity (10, 11, 14). However, because of the inability to transmit sequences of such size in retrovirus vectors, we chose to attempt to incorporate "core elements" of HS2, HS3, and HS4, which are markedly reduced in size and have been shown to retain at least partial LCR activity, into vectors carrying the β-globin structural gene. The following sequences were employed for our studies, based on studies in transgenic animals (17–22, 27–29): (*i*) the 283-bp *Sac* I–*Ava* I core HS4 fragment (29); (*ii*) the 260-bp core HS3, including the *Hph* I–*Fnu*4HI segment (27, 28) and the upstream NF-E2 site, which encompasses all sites footprinted *in vivo* (45, 46); and (*iii*) a 478-bp *Hin*dIII–*Sna*BI HS2 fragment encompassing all sites footprinted *in vivo* (45, 47) in the core *Hin*dIII–*Xba* I fragment (19–22) and most of the adjacent alternating purine-pyrimidine stretch (21, 48). As shown previously in Fig. 1, one specific orientation of the sites greatly reduced proviral transmission. Accordingly, the three sites were subcloned in such a way that all eight permutations of relative arrangement were generated. The resulting cassettes were introduced, in sense and antisense orientations upstream of the β-globin sequence in the high-titer β-globin vector shown in lanes B of Fig. 1. As shown in Fig. 4, transmission of these vectors shows significant variations with regard to copy number and genomic stability (e.g., the two species transmitted in lanes H). Among these 16 constructs, we identified four combinations which were transmitted at high titer without rearrangements (lanes A, E, L, and N). All bear HS2 in the antisense orientation relative to retroviral transcription. The reason for the lack of the endogenous band in lanes E is unclear.

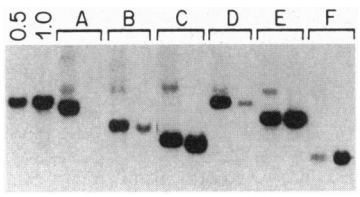

FIG. 3. Comparison of transmission of β-globin sequences in two different vectors. Lanes A–C, pSG vector; lanes D–F, pMFG vector. Lanes A and D, β-globin gene (as in Fig. 1, lanes A); lanes B and E, intron 2 deleted minigene (as in Fig. 2, lanes J); lanes C and F, βCS (as in Fig. 1, lanes B). See Figs. 1 and 2 for nomenclature and methods. Signal intensity between lanes can be directly compared owing to very even loading (data not shown) except for lanes F, which were loaded with 5 μg.

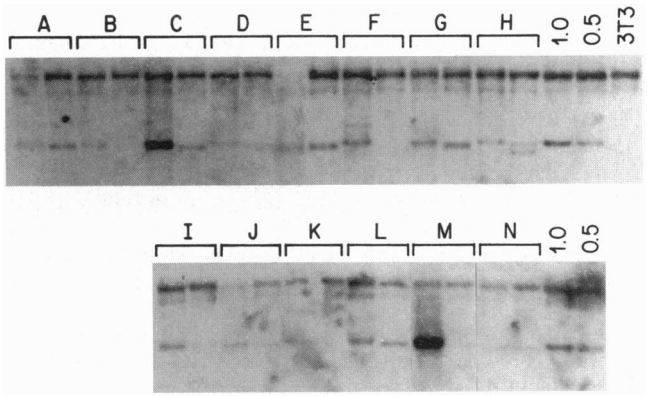

FIG. 4. Effect of HS orientation on vector transmission. Southern blot analysis of packaging cell (ψ-CRE, left lane) and target cell (NIH 3T3, right lane) genomic DNA (see previous figure legends for methods). The top band corresponds to the endogenous globin genes. HS orientation (S, sense; s, antisense) in the vector is (in the order HS4-HS3-HS2): A, Sss; B, reversed A; C, sss; D, reversed C; E, SSs; F, reversed E; G, sSs; H, reversed G; I, SsS; J, reversed I; K, ssS; L, reversed K; M, sSS; and N, reversed M. Lane 3T3, NIH 3T3.

For further studies, we selected the combination with the highest titer and in which HS2 and HS3 were oriented in the same direction as the β-globin gene and adjacent to its promoter. The resulting vector, Mβ6L, is shown in Fig. 5. In summary, this construct contains a 2150-bp *Sna*BI–*Pst*I β-globin fragment including a full intron 1 and a 476-bp intron 2 and lacking the 3′ enhancer [on the basis of other studies that suggest it is redundant in the presence of the LCR (24)]. The 1-kb LCR fragment comprises the HS2, HS3, and HS4 core sequences described above (Fig. 5*A*). Southern blot analysis of NIH 3T3 cells transduced by Mβ6L indicates that approximately one proviral copy is transferred per cell (Fig. 5*B*). On the basis of previous comparisons of proviral copy number and titers measured by selectable marker expression (unpublished results), the titer of Mβ6L is approximately 1×10^6 per ml.

Mβ6L Directs Elevated β-Globin Expression, Detectable at All Integration Sites in MEL Cells. To test the biological activity of Mβ6L, we chose MEL cells as target cells for gene transfer, as those cells have been extensively employed in studies of β-globin transcription (10, 14–16, 23–26). A panel of MEL cell clones infected with Mβ6L was generated by cocultivation of C88 MEL cells and virus-producing cells and subsequent subcloning by limiting dilution (see *Materials and Methods*). Southern blot analyses confirmed that intact vector copies were integrated at different chromosomal positions for each clone (data not shown). Expression of human β-globin was quantitated by RNase protection using total RNA extracted from dimethyl sulfoxide-induced clones. Levels of human β-globin mRNA transcripts were measured by using the phosphorimager and normalized to endogenous α- or β-major globin transcripts. This analysis is summarized in Table 1, where values are normalized to human β-globin expression in Hu11, a MEL cell containing human chromosome 11 under selective pressure (49), based on the comparison of their respective human β to murine α mRNA ratios (calculations based on human β/murine β-major yielded very similar results). In a pool of 10^5 unselected MEL cells infected with the parent vector lacking the LCR, Mβ6, the mean level of expression was 2.2% of that measured in Hu11. Mean expression was 78% in MEL cell pools infected with Mβ6L. Human β-globin transcripts were detected in NIH 3T3 fibroblasts but at levels about 1/100 of the level measured from the equal amount of RNA from induced MEL cells (Table 1). This

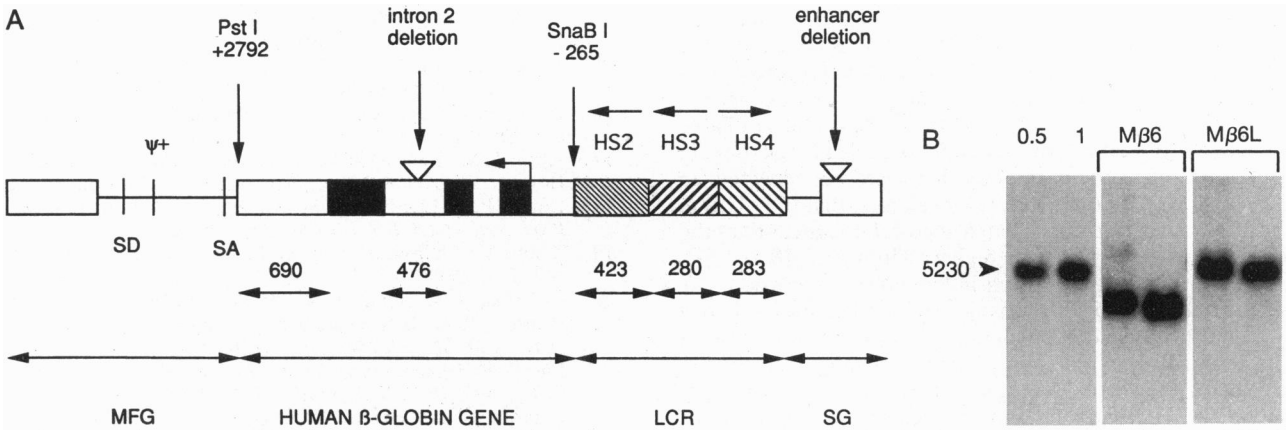

FIG. 5. (*A*) Schematic representation of the Mβ6L vector. Black boxes represent the β-globin exons. Arrows above the HS boxes indicate their orientations. Numbers below delineated segments represent fragment length in bp. ψ+, packaging signal. White boxes at ends represent LTRs. See text for exact sequences. SD, splice donor; SA, splice acceptor; MFG, 5′ sequences derived from the MFG vector; SG, 3′ sequences derived from the SG vector. (*B*) Transmission characteristics of vectors Mβ6L and Mβ6, the parent β-globin gene vector lacking the LCR fragment. Southern blot analysis is carried out as in the previous figures. Length on left is in bp.

finding is consistent with other studies of β-globin expression in transfected fibroblasts (14, 15, 23, 24). In subcloned MEL cells, the expression values ranged from 4% to 146% (ML1–ML7, Table 1). The level measured for each clone was very stable upon repeated measurement as well as upon reinduction. Importantly, in this set of clones and others with slightly different β-globin gene boundaries (MS and RCM, unpublished observations), which represent a total of 17 clones, human β-globin expression, albeit variable in amount, was readily detected in all cases. This is in contrast to clones infected with the vector lacking LCR sequences (Mβ6), in which human β-globin expression was undetectable in 3 of 4 clones (M1–M4; Table 1).

Table 1. Human β-globin expression in dimethyl sulfoxide-induced MEL cells and in NIH 3T3 fibroblasts

Cell	LCR	Huβ gene copy number	Huβ RNA/Mα RNA per vector copy, %
MEL	−	0	0
Hu11	+	1	100
MEL/Mβ6	−	0.5	2.2
MEL/Mβ6L	+	1.6	78
3T3/Mβ6	−	1.9	0.2
3T3/Mβ6L	+	1.8	0.6
M1	−	1	2
M2	−	1	<0.1
M3	−	1	<0.1
M4	−	1	<0.1
ML1	+	1	61
ML2	+	1	101
ML3	+	1	146
ML4	+	1	4
ML5	+	3	28
ML6	+	4	35
ML7	+	5	74

Protected RNA transcripts were fractionated on a 6% polyacrylamide/urea gel and their radioactivities were measured by using the phosphorimager. Results are expressed as human β-globin mRNA/endogenous murine α-globin mRNA divided by vector copy number, normalized to the values measured in the Hu11 clone (see text). Values in NIH 3T3 cells are expressed as human β-globin RNA divided by vector copy number in 1 μg of total RNA normalized to the human β-globin signal in 1 μg of total RNA from dimethyl sulfoxide-induced Hu11 cells. In the first six lines, data from a large polyclonal cell population; in the rest of the table, data from individual MEL cell clones.

DISCUSSION

In this study, we report the resolution of two major obstacles to the implementation of retrovirus-based genetic treatment of β-globin disorders: first, the generation of a high-titer retroviral vector suitable for obtaining the efficient transduction of hematopoietic stem cells and, second, the generation of vectors capable of expressing high levels of the β-globin gene in an erythroid-specific fashion. Our systematic study of vector transmission indicated problems associated with incorporation of both the β-globin structural gene itself and the LCR core sequences. In the case of the β-globin structural gene, our results parallel those of Miller *et al.* (43), who had observed that the poor transmission of retroviral vectors carrying the β-globin structural gene was caused by both untranslated sequences in the 5′ and 3′ regions of the *Hpa* I–*Xba* I β-globin fragment and sequences within intron 2. Our results differ slightly from those of that study in that we found that sequences within intron 2 alone primarily account for the poor transmission of β-globin vectors (Fig. 2). We found that features of retroviral vector design also affected proviral transmission of β-globin sequences. For example, modifications of the vector sequences juxtaposed to the 3′ end of the β-globin gene indicated that including the Moloney MLV splice acceptor region from pMFG (34) resulted in a 5-fold increase in titer. Deletion of the U3 region of the 3′ LTR (33) did not decrease titer and, in fact, led to a moderate (2-fold) increase (data not shown).

We also confirmed the studies of Novak *et al.* (30) that suggested that LCR sequences can lead to significant vector instability (data not shown). In our studies, even the core fragments incorporated together led to decreased transmission and vector instability in particular sequence arrangements (Figs. 1 and 4). To overcome this problem, we examined the transmission of all 16 permutations of the core sites, and we identified combinations that result in stable transmission and the highest titers (Fig. 4, lanes A, E, L, and N).

The LCR was first functionally defined *in vivo* in transgenic mice by using a 20-kb LCR fragment (10, 11), a 6.5-kb fragment (15), or single HS sites (17–22, 27–29). Studies in animals suggested that the LCR confers position-independent expression, based on the calculation that β-globin expression per transgene copy is relatively constant (7). However, it should be noted that transgenic studies involving either complete LCR or core sequences generally relied on the analysis of cells possessing more than one copy per cell. In contrast, our use of retrovirus-mediated gene transfer made it possible to examine the issue of position independence under conditions

where one copy of the transcription unit per cell is integrated in a precise reproducible way and in the absence of any selection (50). In a panel of MEL cell clones representing independent integration sites, we found that expression was detectable in all clones, yet ranged from 4% to 146% (Table 1) of human β-globin expression in the Hu11 cell line in single-copy clones (the variability decreases, as expected, in multicopy clones). Our data therefore suggest that the LCR, in this configuration, acts more like a classical enhancer than the proposed function of an LCR (reviewed in refs. 7–10 and 51). The absence of strictly position-independent expression (10, 14, 15) we observe raises the issues of whether the LCR can truly confer position-independent expression when present at one copy per cell, whether the compact arrangement of the transcription and chromatin regulators within our vector leads to suboptimal interactions and inefficient LCR activity, or whether it is specifically the combination of core elements which we have employed which lack this ability (see refs. 17–22 for HS2, 27 and 28 for HS3, and 29 for HS4). Whatever the explanation, our data strongly suggest that it is important to carefully reevaluate the characteristics of larger LCR-containing sequences in the context of single-copy insertions.

For the purposes of gene therapy the most critical test of the LCR sequences will be the introduction of the virus genomes described above into hematopoietic cells followed by their transplantation. The inability to obtain chromosome position-independent expression of the human β-globin gene in the context of single-copy insertions would make the prospects for effective genetic treatment of hemoglobinopathies less likely than previously believed.

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