Retroviral vector sequences inhibit human β -globin gene expression in transgenic mice

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Received July 19, 1994; Revised and Accepted September 15, 1994

ABSTRACT

The DNase I hypersensitive site 5' HS2 of the human β -globin locus control region confers position-independent, high-level expression on the human β -globin gene in transgenic mice. When a 5' HS2 β -globin construct is flanked by retroviral vector sequences derived from Moloney Murine Leukemia Virus (MoMLV), expression of the β -globin gene is severely inhibited. Apparently, one or more elements within the MoMLV genome is capable of repressing transcription of the human β -globin gene in transgenic mice. A construct lacking the retroviral enhancer also fails to express the β -globin gene, indicating that this region of the virus is not essential for repression. Further analysis may permit the identification of specific viral sequences that inhibit gene expression; these sequences could then be deleted or mutated to produce improved viral vectors.

INTRODUCTION

The region of DNA containing the four erythroid-specific, DNase I hypersensitive sites located 11–18 kilobases upstream of the human ϵ -globin gene is required for high-level expression of the ϵ -, γ - and β -globin genes (Fig. 1A). This region has consequently been designated the Locus Control Region (LCR). When the entire LCR is cloned upstream of human globin genes and these constructs are microinjected into fertilized mouse eggs, high-level erythroid-specific expression is observed in the transgenic animals that develop (1, 2). Expression of LCR β -globin constructs is enhanced 300-fold over expression of the β -globin gene without LCR sequences. In addition, all of the transgenic mice that contain an intact copy of the LCR β -globin transgene express correctly initiated human β -globin mRNA. These results indicate that the LCR performs the dual functions of enhancing β -globin gene expression and overcoming chromosomal position effects associated with the site of transgene integration.

Our laboratory and the laboratories of others have demonstrated that a single DNase I hypersensitive site of the LCR, 5' HS2, is sufficient to convey enhancer activity and position-independent expression on a human β -globin gene in transgenic mice (2–8).

A 1.1 kb *KpnI*–*XbaI* fragment of 5' HS2 enhances β -globin gene expression 75-fold and provides position-independent expression in all mice containing an intact transgene (5, 9). In this report, we describe the effects of flanking a construct composed of a 1.1 kb (*KpnI*–*XbaI*) 5' HS2 site and a 4.1 kb (*HpaI*–*XbaI*) human β -globin gene with sequences obtained from the Moloney Murine Leukemia Virus (MoMLV) vector LXS_N (10). Surprisingly, expression of the human β -globin gene was severely inhibited. Apparently, MoMLV sequences are capable of overcoming the positive effects of the LCR on human β -globin gene expression in transgenic mice. This finding may be applicable to the more general problem of sustaining expression of retrovirus-transduced genes in primary tissues (11, 12).

MATERIALS AND METHODS

Cloning of 5' HS2 β -globin constructs for microinjection

The retroviral vector LXS_N (10) was modified by the removal of the SV40 promoter and neomycin phosphotransferase gene. This deletion was accomplished by digestion of a plasmid containing the vector with the enzymes *Bam*HI and *Rsr*II which resulted in a deletion of 1048 bases. The resulting vector contained the original MoMLV sequence and the 5' LTR from Moloney Murine Sarcoma Virus (MoMSV). The 1.1 kb 5' HS2 site (*KpnI*–*XbaI* fragment) and 4.1 kb β -globin gene (*HpaI*–*XbaI* fragment) were cloned into the polylinker site of the retroviral vector in reverse orientation relative to the 5' LTR. For microinjection, the fragment LTR β HS2 LTR was digested with *KpnI* and was purified from plasmid sequences (Fig. 1B). A 1.0 kb fragment of the retroviral vector containing the 3' part of R, U5, ψ , and *gag* is located upstream of HS2 β -globin. A 0.7 kb fragment of the vector containing U3 and the 5' part of R is downstream. This fragment forms the complete retroviral LTR on either side of the HS2 β -globin construct following transgene integration in head-to-tail arrays (Fig. 1C). As a control, a *Bgl*II fragment which contained only HS2 β was prepared from the same plasmid (Fig. 1B). A third construct was made in which a *NheI*–*SacI* fragment of the retroviral vector was deleted (Fig. 1B). This deletion removed the retroviral enhancer and the promoter distal to the TATA box. A fourth

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A

B

C

construct was made in which a second 1.1 kb 5' HS2 site was cloned downstream of the β -globin gene in the original plasmid (Fig. 1B).

Microinjection and analysis of transgenic mice

Fragment preparation and microinjection were as described (2, 13). Transgenic animals were identified by dot blot hybridization. Copy number and integration of the transgenes in head-to-tail arrays was confirmed by Southern blot analysis which demonstrated intact transgenes of the following sizes: LTR β HS2 LTR, 6.9 kb; β HS2, 5.2 kb; LTR β HS2 LTR (Δenh), 6.5 kb; LTR HS2 β HS2 LTR, 8.0 kb.

Analysis and quantitation of β -globin gene expression

Expression was analyzed by primer extension and further quantitated by solution hybridization (2, 13). Solution

hybridization controls for variations in primer-specific activity and differences in annealing efficiencies by quantitation against standard curves. The limit of detection of the human β -globin RNA by the solution hybridization assay is 1.0 pg. This value corresponds to 3 molecules/cell assuming 20 pg of RNA \ fetal liver cell and 20 μ g of total RNA in the hybridization. Percent endogenous expression is calculated as (human β -globin mRNA/murine β -globin mRNA) $\times$ 100%. Percent expression per gene copy is calculated as [(human β -globin mRNA/human β -globin gene)/(murine β -globin mRNA/murine β -globin gene)] $\times$ 100%.

RESULTS AND DISCUSSION

Fertilized mouse eggs were microinjected with the constructs illustrated in Fig. 1B and implanted into the uteri of foster mothers. After 16 days of development, fetuses were removed and the fetal livers were tested for expression of the human 5' HS2 β -globin constructs. Expression of the four HS2 β -globin constructs was analyzed by primer extension (Fig. 2) and quantitated by solution hybridization analysis (Table 1). As described in the Materials and Methods section, the sensitivity of the solution hybridization assay permits the detection of as few as three molecules of mRNA per cell. The percent per gene copy expression relative to the mouse β -globin genes is shown together with the number of integrated transgenes as determined by Southern blot analysis (Table 1).

Human β -globin gene expression is inhibited in mice containing the transgene LTR β HS2 LTR in which HS2 β -globin is flanked by retroviral vector sequences. This inhibition of expression is dramatic; no β -globin expression can be detected in mice even with 50 copies of the transgene. Mice that contain the control construct β HS2, which was prepared from the same plasmid as the construct flanked by retroviral vector sequences, express at a level of 27% per gene copy, which is nearly identical to previously published results (9).

An initial attempt was made to determine which retroviral vector sequences were responsible for inhibiting human β -globin gene expression. The construct LTR β HS2 LTR (Δenh) was analyzed in transgenic mice. This construct contains a deletion of the retroviral enhancer and promoter that is similar to the self-inactivating or SIN vector (14). The particular construct that was microinjected contains a slightly larger deletion of retroviral vector sequence than the SIN vector. The sequence which was removed from the construct LTR β HS2 LTR (Δenh) consists of a 383 base *NheI*–*SacI* fragment that includes the entire retroviral enhancer and most of the promoter distal to the TATA box (Fig. 1B). Human β -globin gene expression was undetectable

Figure 2. Primer extension analysis of 5' HS2 β -globin constructs in transgenic mice. For primer extensions, human and mouse β -globin-specific oligonucleotides were end-labeled with [γ - 32 P]ATP (3000 Ci/mmol) and hybridized together with 0.5 μ g of human reticulocyte RNA (lane 1) or 5 μ g of mouse fetal liver RNA (lanes 2–14) and then extended with reverse transcriptase to map the 5' ends of the human and mouse β -globin mRNA. The products were electrophoresed on 8 M urea/8% polyacrylamide gels and autoradiographed for 24 h at -70°C with an intensifying screen. The authentic human β -globin primer extension product is 98 nucleotides and the correct mouse β -globin product is 53 nucleotides. Markers are end-labeled *HpaII* fragments of the plasmid pSP64. Copy numbers for each construct are listed from left to right as the samples appear in the figure: LTR β HS2 LTR (lanes 3–5): 45, 50, and 50; β HS2 (lanes 6–8): 10, 40, and 50; LTR β HS2 LTR (Δenh) (lanes 9–11): 12, 15, and 30; LTR HS2 β HS2 LTR (lanes 12–14): 5, 20, and 20. The three highest copy number samples for each construct were chosen for primer extension analysis.

Figure 1. Human β -globin locus and microinjection constructs. (A) The human β -globin locus on chromosome 11 is illustrated at the top of the figure. Black boxes represent individual globin genes and arrows identify erythroid-specific, DNase I hypersensitive sites that flank the locus. A 1.1 kb *KpnI*–*XbaI* fragment containing the 5' HS2 sequence was inserted immediately upstream of a 4.1 kb *HpaI*–*XbaI* human β -globin gene. This 5.2 kb construct was then cloned into the polylinker of the MoMLV-based retroviral vector LXSNI. (B) 5' HS2 β -globin constructs microinjected to produce transgenic mice. A 1.0 kb fragment of the retroviral vector containing the 3' part of R, U5, ψ , and *gag* is located upstream of HS2 β -globin. A 0.7 kb fragment of the vector containing U3 and the 5' part of R is downstream. In LTR β HS2 LTR (Δenh), a 383 base (*NheI*–*SacI*) deletion removes the retroviral enhancer from the construct. (C) Microinjection fragment LTR β HS2 LTR and transgene integration into the host genome. LTR β HS2 LTR was digested with *KpnI* and purified from vector sequences prior to microinjection. The fragment used for microinjection is shown at the top of the figure. Below, three copies of the transgene in a head-to-tail array are illustrated. Integration of the transgenes in head-to-tail arrays was confirmed by Southern blot analysis which demonstrated intact transgenes of the following sizes: LTR β HS2 LTR, 6.9 kb; β HS2, 5.2 kb; LTR β HS2 LTR (Δenh), 6.5 kb; LTR HS2 β HS2 LTR, 8.0 kb. Retroviral vector sequences are located both upstream and downstream of HS2 β -globin as would occur following viral integration into the host chromosome. Restriction sites are as follows: K, *KpnI*; Bg, *BglII*; N, *NheI*; S, *SacI*.

Table 1.

Construct	Copy Number	Percent Expression	Percent Expression per Gene Copy
LTR 8 HS2 LTR	0.5	0	0
	0.5	0	0
	0.5	0	0
	1	0	0
	1	0	0
	1	0	0
	1	0	0
	1	0	0
	1	0	0
	1	0	0
	2	0	0
	2	0	0
	4	0	0
	4	0	0
	4	0	0
	5	0	0
	10	0	0
	40	0	0
	45	0	0
	50	0	0
	50	0	0
8 HS2	0.5	3.6	28.8
	1	18.9	75.4
	1	19.0	75.9
	2	4.1	8.2
	2	15.7	31.3
	3	10.5	14.0
	5	12.5	10.0
	10	28.8	11.5
	40	67.0	6.7
	50	78.8	6.3
LTR 8 HS2 LTR (Δ enh)	0.5	0	0
	1	0	0
	2	0	0
	2	0	0
	4	0	0
	8	0	0
	10	0	0
	12	0	0
	15	0	0
	30	0	0
LTR HS2 8 HS2 LTR	1	0.1	0.5
	2	0.2	0.4
	5	0	0
	20	0.1	0.02
	20	0	0

in these animals, indicating that the retroviral enhancer and distal promoter are not essential for repression.

An additional construct, LTR HS2 β HS2 LTR, that contains a second 1.1 kb 5' HS2 site inserted downstream of the β -globin gene was tested (Fig 1B). Globin gene expression could be detected in 3 of 5 transgenic mice; however, expression averaged only 0.3% per gene copy. Therefore, flanking the β -globin gene with 1.1 kb fragments of the 5' HS2 site provided only marginal protection of the gene from the transcriptional repression associated with the retroviral vector sequences.

Current work is directed at identifying the exact retroviral vector sequence or sequences which are responsible for transcriptional repression. One advantage of the use of transgenic animals in these experiments is that functional retrovirus does not have to be produced. Hence, the retroviral vector can be dissected into smaller fragments and deletions of critical elements can be analyzed. When inhibitory sequences are identified, mutations that reverse repression but do not inhibit retroviral replication may be defined.

Expression of genes flanked by sequences derived from other viral vectors is being examined. Perhaps vectors derived from viruses other than MoMLV will demonstrate less transcriptional repression. Additional genes are also being analyzed to determine whether transcriptional repression is gene-specific. Also, insulator sequences such as those of the chicken lysozyme domain and the chicken β -globin locus may overcome the transcriptional repression associated with retroviral vector sequences (15, 16). Insertion of these sequences between LTRs and transduced genes may provide a boundary that insulates genes from repression. The ability of sequences to reduce or eliminate the inhibition associated with retroviral vector sequences could be rapidly assayed in transgenic mice using constructs similar to those described in this paper.

The use of transgenic animals effectively separates the observed transcriptional repression from any process associated with viral infection of cells. The inhibition seen in the transgenic mice which we have produced occurs solely as a function of the retroviral DNA flanking the HS2 β -globin gene constructs. We propose that the transgenic assay described here is a useful screening tool for the identification of viral sequences which may result in decreased expression of transduced genes. Once identified, these inhibitory sequences could be removed or mutated to produce improved viral vectors.

ACKNOWLEDGEMENTS

The University of Alabama at Birmingham Transgenic Animal Core Facility produced the animals described in this paper. The work was supported in part by grants HL 43508 and HL 35559 from the National Institutes of Health. S.L.M. is supported by the UAB Medical Scientist Training Program.

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