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Position-independent human β-globin gene expression mediated by a recombinant adeno-associated virus vector carrying the chicken β-globin insulator

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Abstract The position-independent expression of transgenes in target cells is an essential subject for determining effective gene therapies. The chicken β-globin insulator blocks the effects of regulatory sequences on transcriptional units at differential domains. We prepared a recombinant adeno-associated virus (rAAV) containing various combinations of the DNase I-hypersensitive site 2 (HS2), 3 (HS3), and 4 (HS4) core elements from the human β-globin locus control region (LCR), the human β-globin gene, and the herpes virus thymidine kinase promoter driven neomycin-resistant gene (*neo*^{R}) (rHS432, rHS43, rHS42, rHS32, and rHS2), and also rAAV containing two copies of the 250-bp core sequence of the chicken β-globin insulator on both sides of the rHS2 (rIns/HS2/2Ins). After isolating neomycin-resistant mouse erythroleukemia (MEL) cells infected with each rAAV, we analyzed the rAAV genome by Southern blots and polymerase chain reaction (PCR), using primers specific for HS core elements and the human β-globin gene. All clones contained a single copy of the rAAV genome in the chromosome, however, some of them had a rearranged proviral genome. In five clones with a single unrearranged rAAV genome for each rAAV construct, we assayed the expression of the human b-globin gene relative to the endogenous mouse β^{maj}-globin gene, using quantitative reverse transcriptase (RT)-PCR. In clones infected with rHS432, the expression level of the human β-globin gene ranged from 51.6% to 765.6% of that in the mouse β^{maj}-globin gene. Likewise, in rHS43, the expression level ranged from 36.7% to 259.0%; in rHS42, from 47.8% to 207.0%; in rHS32, from 47.9% to 105.4%; and in rHS2, from 6.1% to 172.1%, indicating a high variability of expression level in clones infected with recombinant virus lacking the insulator. In contrast, in clones infected with rIns/HS2/Ins, the range of expression of the human β-globin gene ranged from 52.8% to 58.3% of that in the mouse β^{maj}-globin gene. These results indicate that the insulator functioned dramatically to reduce the variability of transgene expression due to the position effect. This insulator-rAAV vector system holds promise to provide a constant level of transgene expression for gene therapy, regardless of the insertion sites on the chromosome.

Key words Gene therapy · β-Globin gene · Adeno-associated virus · Position effect · Insulator

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Introduction

Thalassemias are a group of inherited anemias characterized by the reduced production of globin chains (Weatherall and Clegg 1981). This syndrome occurs worldwide. Patients with severe phenotypes require erythrocyte transfusions that can be associated with life-threatening iron overload, despite intensive chelation (Wolfe et al. 1985). Bone marrow transplantation has been performed with some success, but this treatment is feasible in only a small percentage of affected patients (Lucarelli et al. 1990; Ferster et al. 1992). Pharmacologic approaches are intended to increase γ-globin gene transcription, leading to more effective erythropoiesis and/or decreased hemolysis in patients with β-thalassemia (Ley et al 1982). However, these treatments are potentially toxic, with unknown long-term complications. Gene addition strategies are rational approaches to the treatment of thalassemia syndromes. In early experiments, retrovirus-mediated gene transfer revealed that expression of the human β-globin gene was erythroid-specific, but insufficient for gene therapy application because expression of the human β-globin transgene was extremely low and integration site-dependent (Cone et al. 1987; Dzierzak et al. 1988). The prospects for increasing β-globin transgene expression to therapeutic levels were

greatly improved by the discovery of the locus control region (LCR) of the human β-like globin gene cluster. The LCR confers erythroid-specific and elevated expression on a gene linked in cis. Some studies demonstrated that LCR sequences provided for chromosomal position-independent expression of linked genes, thus distinguishing them from classical enhancers (Grosveld et al. 1987; Ryan et al. 1989; Talbot et al. 1989; Blom van Assendelft et al. 1989). The LCR contains four DNase I-hypersensitive sites (HS) located 50–60 kb upstream of the human β-globin gene. It was subsequently shown that the activity of each HS was localized to core elements (Collis et al. 1990; Ney et al. 1990; Philipsen et al. 1990; Talbot et al. 1990; Pruzina et al. 1991). Expression studies in virally transduced cells showed that juxtaposed core elements of the LCR could enhance expression of the linked globin gene in an erythroid-specific fashion, but failed to reduce variability of expression due to integration sites (Walsh et al. 1992; Miller et al. 1993; Leboulch et al. 1994; Einerhand et al. 1995; Sadelain et al. 1995).

The *Drosophila* specialized chromatin structures (scs/scs') (Kellum and Schedl 1991; Kellum and Schedl 1992) and suppressor of the Hairy wing (su[Hw])-binding regions (Holdridge and Dorsett 1991; Geyer and Corces 1992; Roseman et al. 1993) block enhancer-activated transcription in a position-dependent manner when placed between a gene and enhancer, without inactivating either the promoter or enhancer (Kellum and Schedl 1992; Holdridge and Dorsett 1991; Geyer and Corces 1992; Cai and Levine 1995; Scott and Geyer 1995). These insulators can confer position-independent expression on transgenes, when present in flanking positions, presumably by blocking effects from surrounding chromatin (Kellum and Schedl 1991; Roseman et al. 1993). An insulator role has also been demonstrated in the chicken globin LCR DNase I-hypersensitive site 4 (HS4) region (Chung et al. 1993) and the chicken lysozyme LCR A element (Bonifer et al. 1994). When the chicken HS4 region was tested in enhancer-blocking colony assays, the result was a reduction in the number of drug-resistant colonies when the selectable marker was flanked by these sites (Chung et al. 1993). The chicken HS4 region was also tested with the *white* gene in *Drosophila* and showed insulating properties (Chung et al. 1993) similar to those observed in the *Drosophila*, scs and scs' border elements. Most of the insulating activity of the chicken HS4 region resides in a 250-bp core region with multiple protein binding sites (Chung et al. 1997) and is located in a CpG island corresponding to the region of DNase I hypersensitivity (Chung et al. 1997).

One of final goals of genetic treatment for thalassemia syndromes is to introduce a functional globin transcription unit in hematopoietic stem cells and to express the transgene in a manner that is erythroid-specific, elevated, position-independent, and sustained over time. The present study was an attempt to elucidate the position effect of transduced gene expression. We selected a recombinant adeno-associated virus (rAAV)-mediated gene delivering system, for the following reasons: AAV is nonpathogenic (Berns and Bohenzky 1987), integration of the viral genome is efficient (Samulski et al. 1991; Kotin et al. 1992), virus integration appears to have no apparent effect on cell growth or morphology (Handa et al. 1977), there is a highly efficient transduction of human hematopoietic progenitor cells (Goodman et al. 1994), and there is no possibility of a rearranged proviral genome due to unexpected splicing events commonly observed in the globin gene retroviral delivery system (Novak et al. 1990). We constructed a series of rAAV vectors containing the human β-globin gene and various combinations of HS core elements, and another rAAV vector containing two copies of the 250-bp core fragment of the chicken β-globin insulator in both sides of the HS-globin gene cassette. We observed consistent levels of expression of the human β-globin gene in the MEL clone derived by infecting only rAAV with the insulator, indicating that this optimized vector can reduce the variability of transgene expression after random insertion of viral vectors. The insulator could be highly pertinent for gene therapy using a rAAV vector, in combination with potent transcriptional activators.

Materials and methods

Cell culture

Mouse erythroleukemia (MEL) cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 4 mM glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin. All cultures were incubated at 37°C with 5% CO_2, 95% air in a humidified incubator.

Vectors and the helper plasmid

We used psub201, an infectious AAV type2 recombinant plasmid and pAAV/Neo, in which the AAV coding region in psub201 was substituted for the herpes virus thymidine kinase promoter driven neomycin-resistant gene (neo^R). The helper plasmid pAd/AAV containing the AAV *rep* and *cap* genes flanked with adenoviral terminal repeat sequences were kindly obtained by Dr. T. Shenk of the Department of Biology, Princeton University.

Construction of the recombinant adeno-associated virus (rAAV) vector

The six rAAV plasmids we constructed are shown in Fig. 1. Each contains a *Sna* BI-*Swa* I fragment of the human β-globin gene and the neomycin resistance gene, under control of a herpes simplex virus thymidine kinase promoter. The elementary core HS fragments used were HS4 (a 280-bp *Sac* I-*Ana* I), HS3 (a 224-bp *Hph* I-*Fun* 4HI), and HS2 (a 374-bp *Hin* dIII-*Xba* I) fragments obtained by PCR. To facilitate the subcloning of PCR fragments into the vector, nucleotide sequences of PCR primers for HS2 and HS4 were modified by adding the *Pst* I and *Xba* I linkers within the *Xba* I and *Sac* I recognition sequences of sense primers, respectively. The following sets of primers were used for the preparation of each HS fragment: HS4A 5′-GATCTAGA

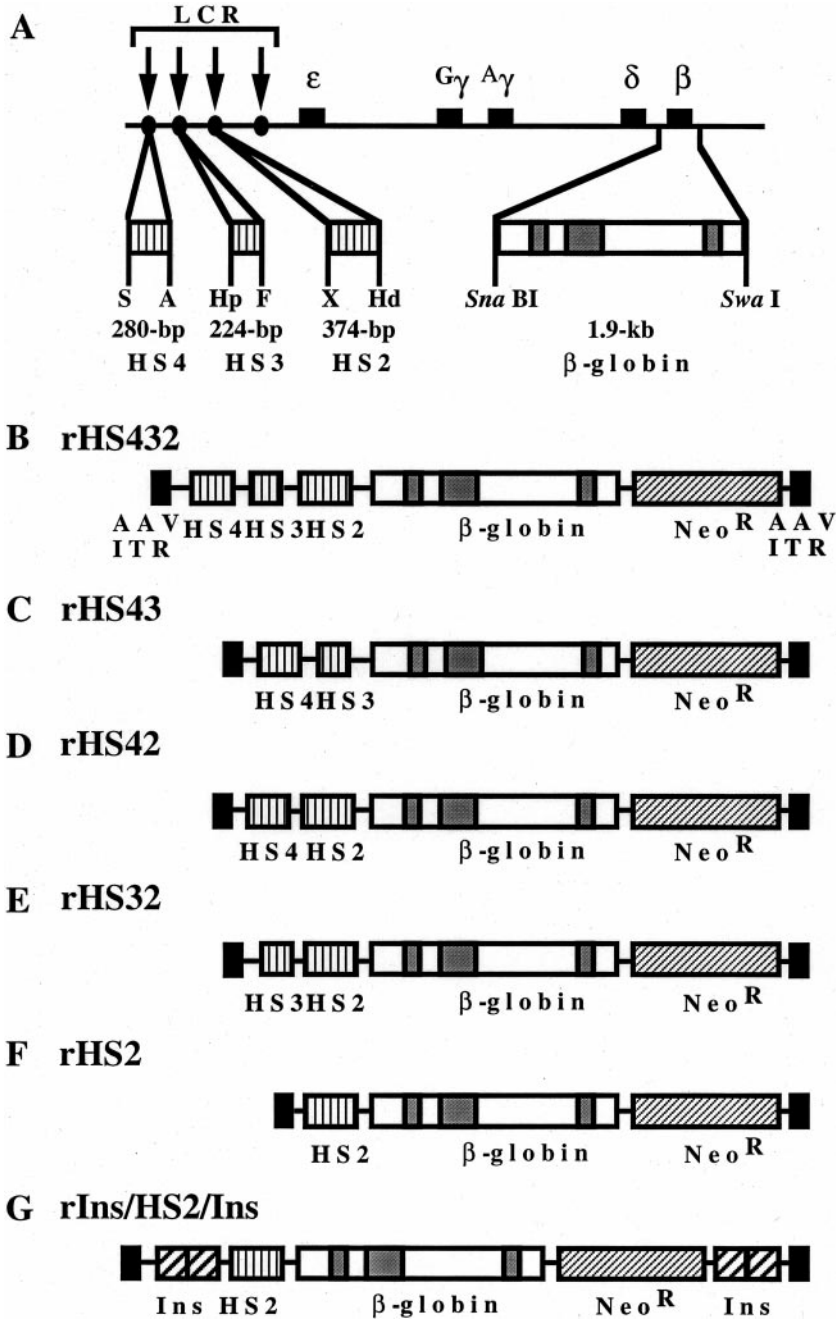

Fig. 1A-F. Structure of the human β-globin cluster and the recombinant adeno-associated vivus (rAAV) plasmid. **A** Structure of the human β-globin gene cluster. The five functional genes (ε, Gγ, Aγ, δ and β) are indicated by *small black boxes*. The position of the DNase I-hypersensitive site (*HS*) regions in the locus control region (LCR) is indicated by *arrows*. HS fragments and the human β-globin gene used in the vector construction are indicated. **B-F** Structure of the rAAV plasmid containing various HS regions. *Gray boxes* indicate three coding regions of the human β-globin gene. Cloning sites used for the insertion of HS regions (S=*Sac* I, A=*Ava* I, Hp=*Hph* I, F=*Fun* 4HI, X=*Xba* I, Hd=*Hin* dIII) and *Sna* BI and *Swa* I in the β-globin gene are shown in **A**. **G** The two copies of the 250-bp fragment of the core sequence of the chicken β-globin insulator (Ins) are inserted on both sides of rHS2 (**F**)

AGCTCTTGGGGACCCCAGTA-3′, including the *Xba* I site starting at the first nucleotide of the *Sac* I recognition sequence, and HS4B 5′-TCACTAGTCTCGGGAATGG-GAGGGAGAG-3′, including the *Spe* I site ending at the *Ava* I site of the HS4 core fragment, HS3A 5′-GGAG-ATCTGGTGACTTTGCGAGCTGGTG-3′, including the *Bgl* II site starting at the *Hph* I site and HS3B 5′-TTGGATCCGCTGCTATGCTGTGCCTCCC-3′, including the *Bam* HI site ending at the *Fun* 4HI site of the HS3 core fragment, and HS2A 5′-GAAATGCATAAGCTT-CAGTTTTTCCTTAG-3′, including the *Nsi* I site starting at the *Hin* dIII site and HS2B 5′-TCCTGCAGCTA-GAATATGTCACATTCTG-3′, including the *Pst* I site ending at the first nucleotide of the *Xba* I recognition sequence of the HS2 core fragment. For PCR, 0.3 μg of human genomic DNA was mixed with a final 0.2 μM concentration of each primer and 0.2 mM of each dNTP in reaction buffer containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, and 0.1% Triton X-100. The mixture was heated at 94°C for 5 min for strand separation. One unit of *Taq* DNA polymerase (Promega, 2Madison, WI, USA) was then added and the following program was used: 94°C × 1 min, annealing for 1 min and 72°C × 1 min for 30 cycles. The annealing temperatures of the sets of HS4A-HS4B, HS3A-HA3B, and HS2A-HS2B primers were 58°C, 59°C, and 55°C, respectively. Amplified HS4, HS3, and HS2

fragments were digested with *Xba* I-*Spe* I, *Bgl* II-*Bam* HI, and *Nsi* I-*Pst* I, respectively, and were then subcloned into the multicloning-sites of pBluescript II SK+ (Stratagene, La Jolla, CA, USA). After the nucleotide sequences of these subclones had been determined by dideoxy methods, these subcloned fragments were digested with appropriate restriction enzymes and inserted between the two *Xba* I sites present in psub201, to generate recombinant viral DNAs. The insulator core sequence was isolated from pJC5-4 as the *Sal* I-*Hin* dIII fragment. pJC5-4 was kindly provided by Dr. G. Felsenfeld, Laboratory of Molecular Biology, National Institutes of Health. This 250-bp fragment was ligated in tandem and inserted in both sides of rHS2. All fragments were subcloned in the same 5′ to 3′ orientation in psub201. Purification was accomplished by a CsCl density gradient formed in a vertical rotor at 52 000 rpm.

The techniques used to produce rAAV were essentially the same as those described by Rolling and Samulski (1995), except that transfection of DNA was done by lipofection into 293 cells. In this study, transfection of DNA was done by calcium phosphate coprecipitation methods into HeLa cells.

Titration of rAAV stocks

To determine the biological titer of recombinant viral stock, dishes containing 80% confluent HeLa cells were infected with each rAAV stock up to 200 ml for 24 h. The infected HeLa cells were trypsinized, and replaced on three of 6 cm dishes. After 24 h, 800 mg/ml G418 (GIBCO-BRL, Gaithersburg, MD, USA) was added to the culture medium, and numbers of G418-resistant colonies were counted 10-14 days after transduction. The order of viral titer was approximately 1 to 5×10^4 cfu/ml.

rAAV-mediated transduction of MEL and clonal selection

Approximately 5×10^5 MEL cells were infected separately with each rAAV stock, at a multiplicity of infection (MOI) of 0.1, and incubated at 37°C for 48 h. Infected MEL cells were washed with phosphate-buffered saline (PBS), and, resuspended in G418 medium at a final concentration of 800 μg/ml. For further purification, G418-resistant cells were plated in semisolid medium containing 1.5% methylcellulose and 800 μg/ml G418. After 7-10 days, several G418-resistant colonies of MEL cells were taken from the cultures, using a Pasteur pipette, and placed in suspension culture.

Analysis of the rAAV genome structure in infected cells

Genomic DNA was extracted from each clone, as described (Sambrook et al. 1989). Organization of the human β-globin gene and HS fragments was examined by PCR, using the specific primers described below. The 5′ part of the human b-globin gene was amplified using human β-globin gene-specific primers EE1 (5′-CTTACATTTGCTTCTGACAC-3′) and EE2 (5′-ACAGATCCCCAAAGGACTCAAAG-3′), located within the region containing the cap site and the second exon of the human β-globin gene, respectively, generating a 334-bp PCR-amplified fragment. Individual HS fragments were amplified using HS-specific primers (HS4A-HS4B), (HS3A-HS3B), and (HS2A-HS2B), which generate 280-bp, 220-bp, and 370-bp fragments, respectively. To determine the genome arrangement and copy number, 10 μg of genomic DNA from rHS432, rHS43, rHS42, and rHS32 clones and rHS2 and rIns/HS2/Ins clones was digested with *Pst* I, *Kpn* I, or *Hin* dIII and *Hin* dIII, *Kpn* I or *Kpn* I and *Hin* cII, respectively. Each DNA sample was fractionated by 0.6% agarose gel electrophoresis in Tris-borate ethylediamine tetracetate (TBE) buffer, transferred to HybondN+ (Amersham, Arlington Heights, IL,USA), and then hybridized with an *Eco* RI-*Bam* HI ^{32}P-labeled fragment of the human β-globin gene. The filters were washed once with 2 × standard saline citrate (SSC)/0.1% sodium dodecyl sulfate (SDS), once with 0.5 × SSC/0.1% SDS, and twice with 0.2 × SSC/0.1% SDS, each at 65°C for 15 min. The hybridized probe was detected on autoradiography. The copy number of the transduced gene in rHS432, rHS43, rHS42, and rHS32 was obtained by densitometry of the β-globin DNA signal of the *Pst* I, *Kpn* I, or *Hin* dIII band of genomic DNA on Southern blots and compared with that obtained for each AAV plasmid DNA and for two copies of the human β-globin gene in the HeLa genome. Likewise, the copy number of the transduced gene in rHS2 and rIns/HS2/Ins was determined by densitometry of the b-globin gene signal of the *Hin* dIII, *Kpn* I, or *Kpn* I-*Hin* cII band derived from the genomic DNA of each clone to the corresponding band of each rAAV plasmid DNA and the HeLa genome.

Expression analysis

Expression of the globin gene was analyzed using quantitative reverse transcriptase-PCR (RT-PCR). Total RNA was extracted from each clone, using Isogen (Nippon Gene, Tokyo, Japan). The RT reaction was performed using an RNA PCR kit (Perkin Elmer, Branchburg, NJ, USA). For RT, 2.5 μg of total RNA was heated at 65°C for 2 min and then placed on ice. Total RNA was mixed with 2.5 μM random hexamers, 1 mM each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 U/μl RNase Inhibitor, and 2.5 U/μl MuLV reverse transcriptase in a total volume of 25 μl, including an RNA specimen. The RT reaction was as follows: One cycle of 42°C for 15 min, 99°C for 5 min, and 5°C for 5 min. PCRs were performed using HE1 (5′-GAGGAGAAGTCTGCCGTTACTGC-3′) and HE2 (5′-CATTAGCCACACCAGCCACCACT-3′) located within the first and second exons of the human β-globin gene, respectively, generating a 403-bp PCR-amplified fragment. ME1 (5′-GCTGAGAAGGCTGCTGTCTCTTG-3′) and ME2 (5′-GGCCCAGCACAATCACGATCATA-3′), specific for the first and second exons of the mouse β^{maj}-globin gene, respectively, were used to generate a 331-bp PCR-amplified fragment. Five μl of the synthesized cDNA was used as the template for PCR, under the following conditions: for the human β-globin gene, 25 cycles of 94°C for 1

min, 65°C for 1 min, and 72°C for 1 min; for the mouse β^{maj}-globin gene, 25 cycles of 94°C for 1 min, 70°C for 1 min, and 72°C for 1 min. PCR products were separated on 2% agarose gels and stained with Vistra Green (Amersham, Arlington Heights, IL, USA) to a final dilution of 1: 10 000 in Tris-EDTA (TE). The fluorochrome intensity of specific fragments was detected using a Fluoro Imager system (Molecular Dynamics, Sunnyvale, CA, USA) and analyzed using Image QuaNT software (Molecular Dynamics). Expression of the transduced gene was assessed as a percentage of the mRNA-derived intensity from the human β-globin gene in relation to that from the mouse β^{maj}-globin gene. The percentages were calculated as follows:

$$\frac{\text{human } \beta\text{-RNA}}{\text{mouse } \beta^{maj}\text{-RNA}} \times \frac{\text{mouse } \beta^{maj} \text{ gene copy no.}}{\text{human } \beta \text{ gene copy no.}} \times \frac{\text{fragment length of mouse RT-PCR}}{\text{fragment length of mouse RT-PCR}}$$

This value was determined for each of three RT-PCR trials, and the mean and SD of the mean were calculated.

Fluorescence in-situ hybridization (FISH)

The techniques used for FISH were essentially the same as those described by Lichter et al. (1988). Briefly, rIns/HS2/Ins clones were cultured in MEM with 1.5 μg/ml, 3-(1-anilinoethylidene)-5-benzylpyrrolidine-2, 4-dione (TN-16) (Wako Pure Chemical Industries, Osaka, Japan) for 2 h to enrich metaphase cells. The cells were treated with hypotonic solution (0.075 M KCl) and fixed with cold methanol-glacial acetic acid (3: 1). Metaphase cells were washed with fixative, dropped on cold slides and air dried. rIns/HS2/Ins plasmids (1.5 μg) were labeled with biotin-16-deoxyuridine triphasphate (dUTP) with a nick translation kit (Boehringer Mannheim, Mannheim, Germany). About 100 ng of labeled probes was mixed with 1.5 μg of mouse Cot-I DNA and 5 μg of salmon sperm DNA. After denaturation of the probe mixture (75°C for 10 min), the probes were preannealed (37°C for 10 min) to block non-specific signals and hybridized to denatured metaphase chromosome specimens prepared from rIns/HS2/Ins clones. After incubation for 48 h and subsequent post-hybridization washes, the specimens were treated with blocking solution (3% bovine serum albumin, 4 × SSC, and 0.1% Tween 20). Biotin labeled probes were amplified using avidin and biotin-conjugated anti-avidin antibodies, and then detected by fluorescein isothiocyanate (FITC)-conjugated avidin (Boehringer Mannheim, Mannheim, Germany) followed by 4,6-diamidino-2-phenylindole (DAPI) counterstaining. Individual signals were collected using a fluorescence microscope coupled to a charged coupled devise (CCD) camera (Axiophot; Zeiss, Jena, Germany) and merged using Probe Vision software (Applied Imaging, Santa Clara, CA, USA).

Results

rAAV-mediated transduction of the human β-globin gene with various combinations of HS fragments

To evaluate the effects of HS regions on β-globin gene expression in the AAV-mediated gene delivery system, we prepared rAAV constructs that contained various combinations of HS core elements linked to the human β-globin gene. MEL cells were infected separately with each rAAV construct, rHS432, rHS43, rHS42, and rHS32, at an MOI of 0.1. Ten single neomycin-resistant clones were isolated from each rAAV construct. For each clone, the presence of an intact HS region and the b-globin gene region was evaluated by PCR (See Materials and methods). The 334-bp specific PCR fragment of the 5′ part of the human β-globin gene was present in all clones. However, the specific PCR fragments of the HS regions were not evident in two, three, one, and four of ten rHS432, rHS43, rHS42, and rHS32 clones, respectively, suggesting that the rAAV genome was rearranged in the host chromosome of these clones (data not shown). To examine integration and the copy number of the rAAV genome, Southern blot analysis was performed, using *Pst* I digested genomic DNA derived from each clone. Restriction digestion with *Pst* I resulted in an unrearranged internal genomic band of a predicted length, 2.2-kb for rHS432, rHS42, and rHS32 and 2.5-kb for rHS43, because *Pst* I cut twice in each construct. All ten clones of rHS432 contained the intact human β-globin gene, while three, two, and one of ten clones contained a rearranged genome, as reflected by a *Pst* I band that deviated in length from that predicted in rHS43, rHS42, and rHS32, respectively. Digestion with *Kpn* I, which cuts once in each rAAV genome, generates a junction fragment, the length of which depends on the integration site. Southern blots of genomic DNA from all clones with the unrearranged rAAV genome of each construct showed that the single *Kpn* I fragments in each clone were of unique size. Representative results of Southern blot analysis of DNA from five rHS432 clones are shown in Fig. 2. To exclude head-to-head tandem integration, we performed Southern blots of genomic DNA digested with *Hin* dIII, which cuts at the 5′ to the b-globin gene. One hybridizing band per transduced clone was also observed using the same probe on a Southern blot of genomic DNA from all clones (data not shown). These observations indicate that a single copy of the rAAV genome integrated at different sites on the chromosome. A single copy of the transduced rAAV genome was also confirmed by densitometric measurement of the signal from each band on Southern blots, compared with that from each rAAV plasmid DNA and the HeLa genome.

Expression of the transduced human β-globin gene

Total RNA was extracted from five clones for each rAAV construct which proved to contain a single and unrearranged vector genome at different sites on the host chromosome. Products of the human β-globin gene, amplified using ME1-ME2 primers, and the mouse β^{maj}-globin gene, amplified

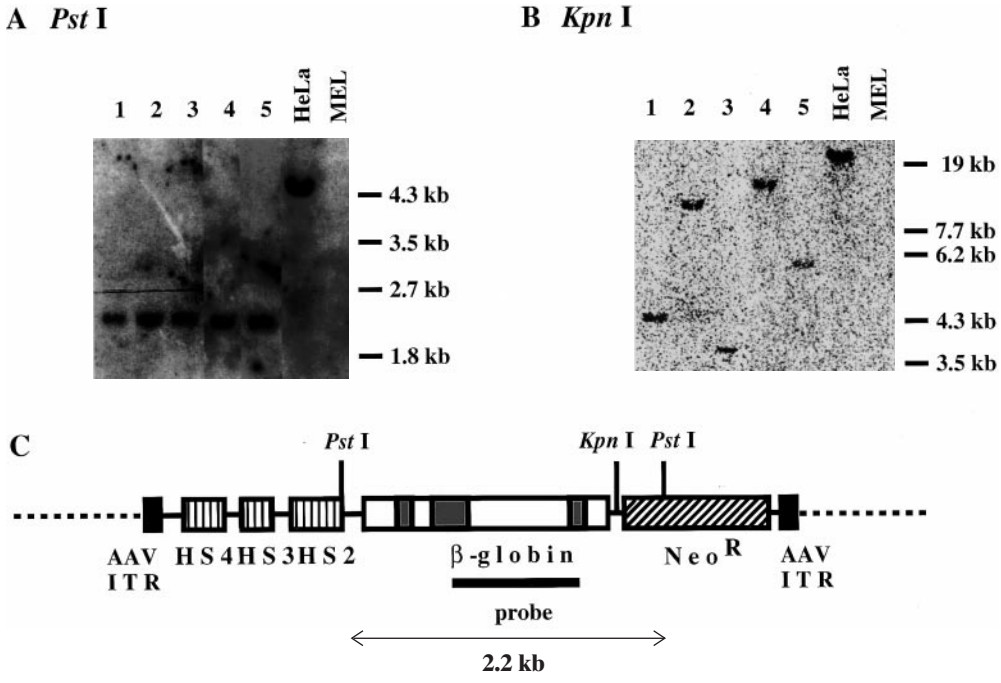

Fig. 2A-C. Southern blot analysis of **A** *Pst* I and **B** *Kpn* I -digested DNA from rHS432 clones. DNAs submitted to Southern blot analysis were extracted from five independent neomycin-resistant clones of rHS432 (lanes *1*, *2*, *3*, *4*, and *5*). *Lane HeLa* contains *Pst* I- or *Kpn* I-digested DNA from HeLa cells, as a positive control, and *lane MEL* contains *Pst* I- or *Kpn* I-digested DNA from mouse erythroleukemia (MEL) cells, as a negative control. The human β-globin probe was prepared by radiolabelling a 917-bp fragment flanked by *Bam* HI and *Eco* RI restriction sites. *Tick marks* indicate the position of the Lambda *Sty* I marker. **C** Schematic of the integrated rHS432 genome. *Pst* I digestion of the integrated rAAV genome results in a 2.2-kb insert containing the human β-globin gene. *Kpn* I digestion generates a junction fragment hybridized with the human β-globin probe

using E1-E2 primers, were not detected in all analyses, indicating the specificity of each primer set in the RT-PCR method. Negative control reaction mixtures contained RT reaction mixtures that were prepared without the addition of RNA. Products of the human β-globin and mouse β^{maj}-globin gene amplified using these samples were not detected in all analyses. To determine the optimal condition for detection and quantitation of human β-globin gene expression, the relative yields of PCR products for the human reticulocytes and MEL cells were determined after various numbers of PCR cycles. We found that the kinetics of amplification of the human β-globin-specific PCR products were similar to those of the mouse β^{maj}-globin-specific products in the human reticulocytes and MEL cells (data not shown). At 25 cycles of PCR, the relative yields of both PCR products were within the linear range of amplification before saturation at the plateau, indicating that it is possible to compare the initial amounts of mRNA template in the human β-globin and mouse β^{maj}-globin cDNA samples (data not shown). An estimate of expression was made as the ratio of human β-globin/mouse β^{maj}-globin gene signal intensity (See Materials and methods). RT-PCR signals are shown in Fig. 3A, C, E, G and are graphically presented in Fig. 3B, D, F, H. The expression per copy of the rAAV genome ranged from 51.6% to 765.6% of a single copy of the mouse β^{maj}-globin gene with a mean expression level of 240.4% in the rHS432 clones. Likewise, the expression level ranged from 36.7% to 259.0% with a mean of 140.7% in the rHS43 clones, 47.8% to 207.0% with a mean of 130.7% in the rHS42 clones; and 47.9% to 105.4% with a mean of 77.6% in the rHS32 clones. Variability in the level of expression was observed in individual clones derived from each rAAV construct, indicating that HS core elements are not capable of conferring position-independent expression to the linked gene.

Effect of the insulator on expression of the β-globin gene

To overcome the position effect variegation we prepared rAAV which contained two copies of the 250-bp insulator core sequence of the chicken β-globin gene in both sides of the HS2, the β-globin gene and the neo gene cassette. Five single neomycin-resistant colonies were isolated from MEL cells infected with rIns/HS2/Ins. Nine clones from rHS2-infected MEL cells, which have no insulator, were also isolated as a control. We examined the integration and structure of the rAAV genome in the host chromosome by Southern blotting and PCR, using primers specific for the β-globin gene and the HS2 region. PCR analysis showed the presence of the transduced human β-globin gene in all clones of rIns/HS2/Ins and rHS2. The HS2 region was detected in seven of nine clones of rHS2 and all of rIns/HS2/Ins. Restriction digestion with *Hin* dIII located within the rIns/HS2/Ins genome released the predicted 3.3-kb unrearranged genomic fragment in all rIns/HS2/Ins clones (Fig. 4A). We also did a Southern blot analysis of genomic DNA digested by both *Kpn* I and *Hin* cII, which cut once and uncut the rIns/HS2/Ins genome, respectively. The results show the presence of fragments of various sizes, indicating that a single copy of the rIns/HS2/Ins genome integrated at different sites on the host chromosome (Fig. 4B). For further evaluation of the integration site of the rIns/HS2/Ins genome, FISH was performed (as described in Materials and methods). A signal of the human β-globin

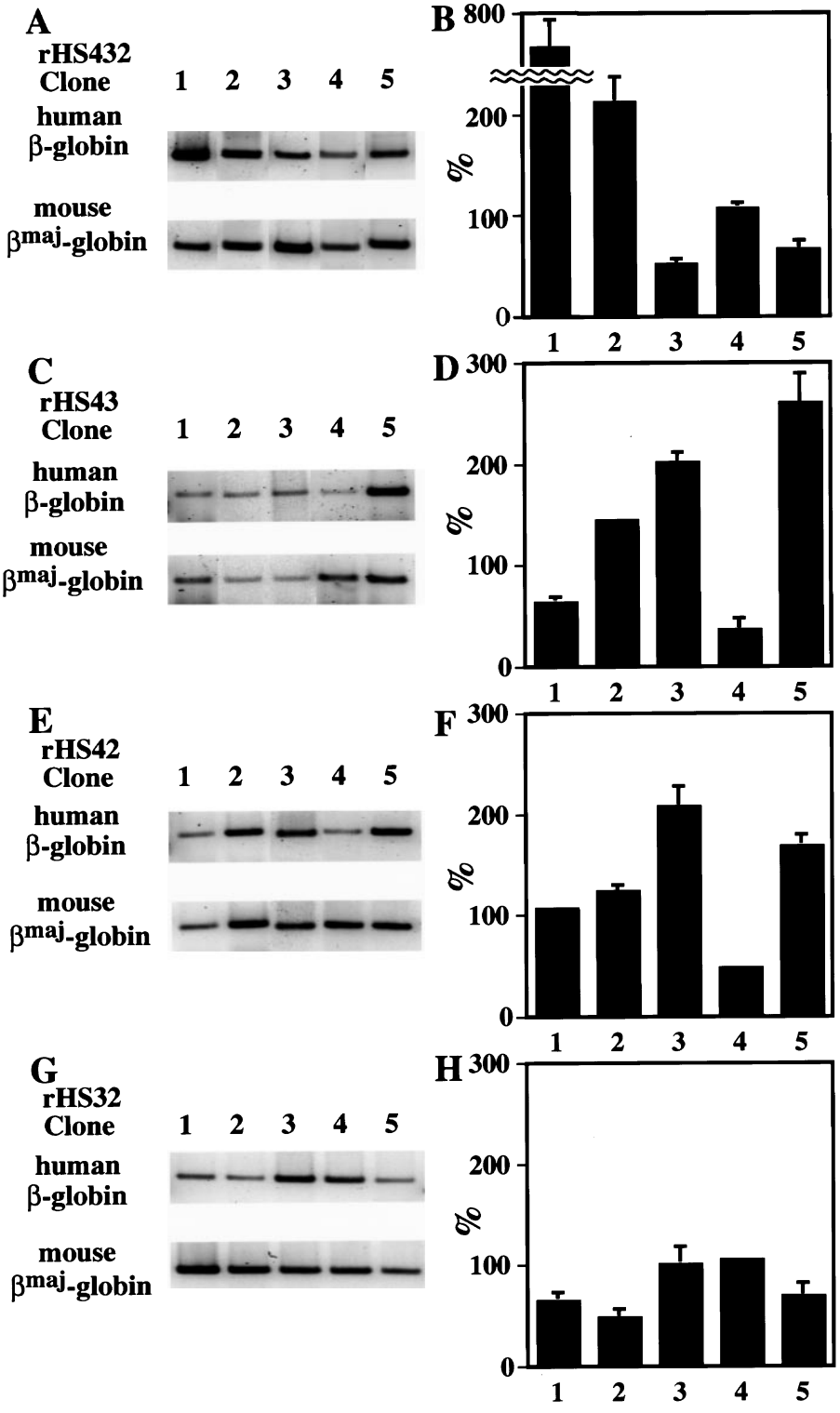

Fig. 3A-H. Expression of the human β-globin gene transduced by rAAV with various combinations of HS regions in MEL clones. Reverse transcriptase-polymerase chain reaction (RT-PCR) products of RNA extracted from five individual clones with a single unrearranged rAAV vector genome for each construct are shown, **A**, **B** rHS432 clones, **C**, **D** rHS43 clones, **E**, **F** rHS42 clones, and **G**, **H** rHS32 clones. The relative amount of human β-globin mRNA is expressed as a percentage ratio of human β-globin RT-PCR products to mouse β^{maj}-globin RT-PCR products. The data represent average values obtained from three independent RT-PCR experiment

gene was detected at a single integration site in all rIns/HS2/Ins clones and each signal was present on a different chromosome. Representative results of FISH analysis of two clones are shown in Fig. 5. These results are in good agreement with the observations of Southern blot analysis. Southern blots of genomic DNA digested with either *Pst* I or both *Kpn* I and *Hin* dIII from nine rHS2 clones showed that seven of the nine had a single and unrearranged rAAV genome at different sites on the chromosome (data not shown). To measure the expression of the human β-globin gene, total RNA was isolated from five clones with a single unrearranged rAAV genome in the chromosome for each construct. RT-PCR analysis was done using specific primers for the human β-globin and mouse β^{maj}-globin genes (See Material and methods) of these five clones. RT-PCR signals are shown and graphically presented in Fig. 6. In rHS2

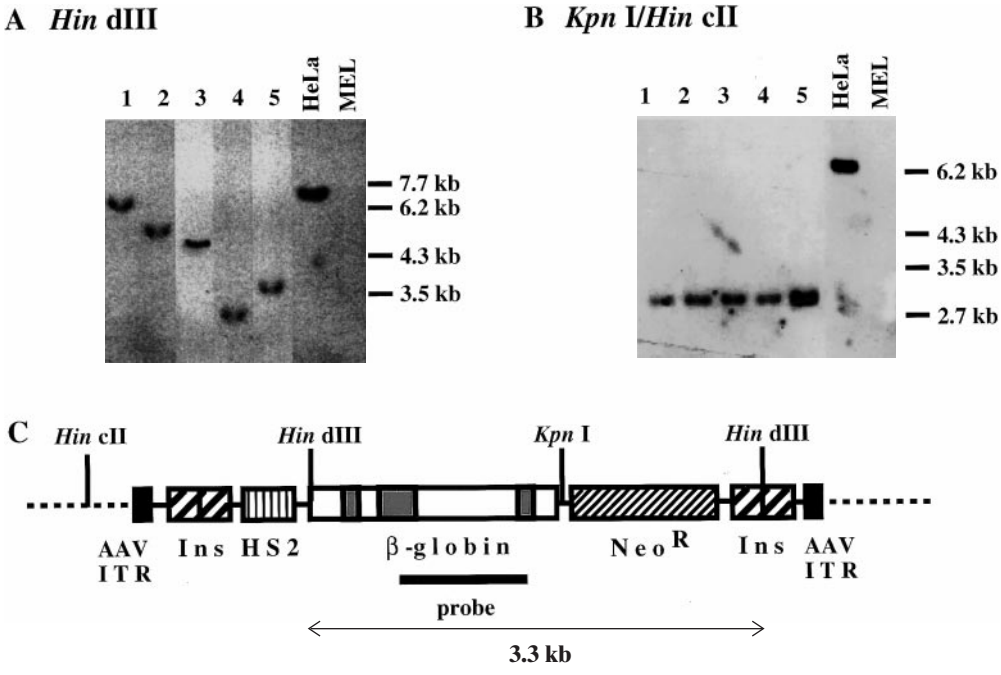

Fig. 4A-C. Southern blot analysis of **A** *Hin* dIII and **B** *Kpn* I and *Hin* cII -digested DNAs from rIns/HS2/Ins clones. DNAs extracted from five independent neomycin-resistant clones of rIns/HS2/Ins were submitted to Southern blot analysis. *Lane HeLa* contains *Hin* dIII- or *Kpn* I and Hin cII-digested DNA from HeLa cells, as a positive control and lane MEL contains *Hin* dIII- or *Kpn* I and *Hin* cII-digested DNA from MEL cells, as a negative control. The human β-globin probe was prepared by radiolabelling a 917-bp fragment flanked by *Bam* HI and *Eco* RI restriction sites. *Tick marks* indicate position of the Lambda *Sty* I marker. **C** Schematic of the integrated rIns/HS2/Ins genome. *Hin* dIII digestion generates a 3.3-kb insert containing the human β-globin gene. Double digestion with *Kpn* I and *Hin* cII results in a junction fragment hybridized with the human β-globin probe

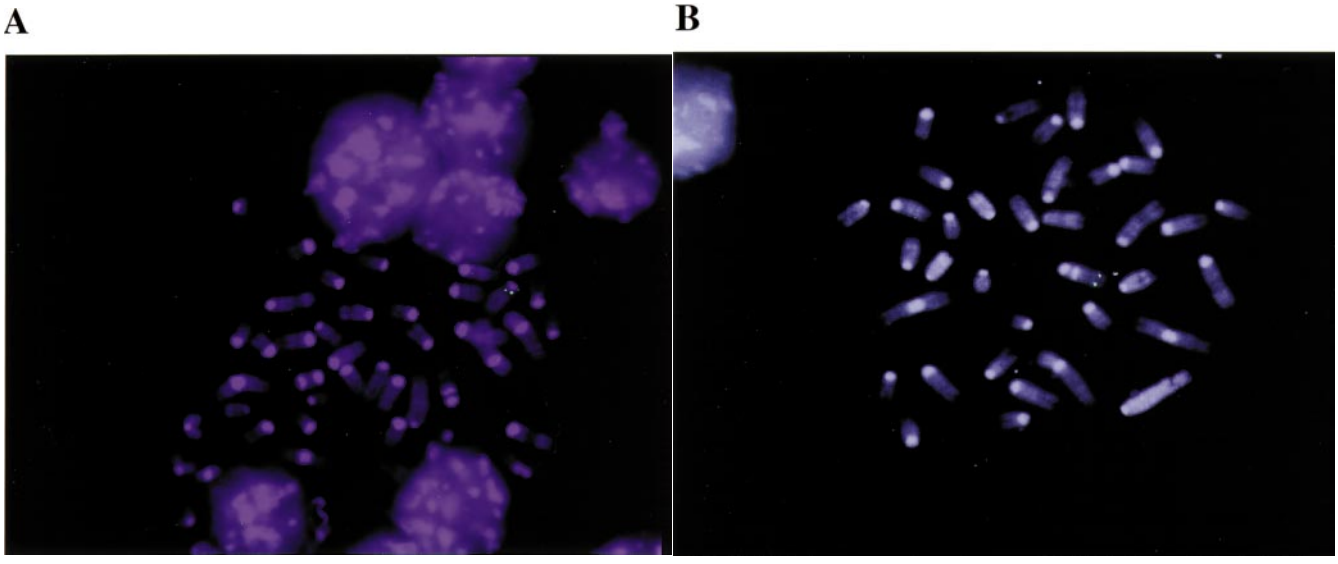

Fig. 5A, B. Fluorescence in-situ hybridization analysis of metaphase chromosome from rIns/HS2/Ins clones. Metaphase chromosomes of two independent rIns/HS2/Ins clones **A** and **B** were hybridized with the biotin-labeled rIns/HS2/Ins probe with subsequent signal detection by fluorescein isothiocyanate (FITC). A single integration of the rIns/HS2/Ins genome was shown at each clone. Each signal of the clones in **A** and **B** was present on a different chromosome. Chromosomes were counterstained with 4-6-diamidino-2-phenylindole (DAPI)

clones, the levels of human β-globin expression ranged from 6.1% to 172.1%, with the mean expression being 51.0% of the endogenous mouse β^{maj}-globin mRNA level. In contrast, rIns/HS2/Ins clones revealed nearly consistent levels of human b-globin mRNA, compared with the mouse bmaj-globin expression (52.8%–58.3%, with a mean expression level of 56.2%).

Discussion

We evaluated the effects of the HS elements of the LCR of the b-globin gene cluster and a chicken β-globin insulator on variability of expression of the β-globin gene transduced by rAAV. The mean expression level of rHS432, rHS43,

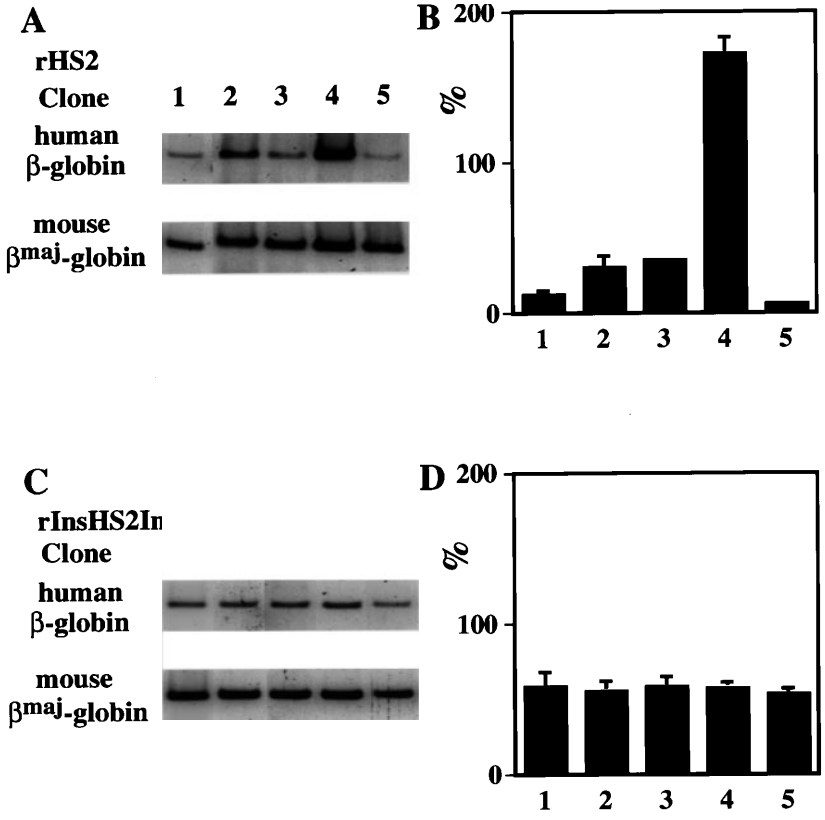

Fig. 6. Expression of the human b-globin gene transduced by rAAV, with and without the insulator in MEL clones. RT-PCR products of RNA extracted from five individual clones with a single unrearranged rAAV vector genome for each construct are shown, A, B rHS2 clones; C, D rIns/HS2/Ins clones. The relative amount of human b-globin mRNA is expressed as a percentage ratio of human b-globin RT-PCR products to mouse bmaj-globin RT-PCR products. The assay was done in triplicate on individual clones

rHS42, rHS32, and rHS2 clones was 240.4%, 140.7%, 130.7%, 77.6%, and 51.0%, respectively, of the level of the mouse β^{maj}-globin expression. The highest mean value was observed in rHS432 clones, indicating that the rAAV vector containing all of HS4, HS3, and HS2 elements rather than HS43, HS42, HS32, or only HS2 elements had a greater effect on increasing expression of the human β-globin gene. However, the human β-globin expression levels varied, irrespective of the combination of HS elements. Expression levels of the transduced human β-globin gene in rHS432, rHS43, rHS42, rHS32, and rHS2 clones varied by more than 14-fold, the range being 51.6%–765.6% relative to endogenous mouse β^{maj}-globin expression, and sevenfold, fourfold, twofold, and 28-fold, respectively. Other investigators have also used AAV vectors with core elements of the human β-globin LCR linked to either the human β- or γ-globin gene (Walsh et al. 1992; Miller et al. 1993; Leboulch et al. 1994; Einerhand et al. 1995; Sadelain et al. 1995). However, the effects of only HS2 and HS432 on globin gene expression were evaluated. Although erythroid-specific expression increased, the expression levels of the transduced human globin gene varied. These results indicate that transcription of the gene with the HS region was not independent of the integration site and was affected by flanking chromatin regions. In transgenic mice, however, the LCR-linked b-globin transgene was expressed in a position-independent and a copy number-dependent manner (Grosveld et al. 1987; Ryan et al. 1989; Talbot et al. 1989; Blom van Assendelft et al. 1989). The discrepancy in position-independent expression due to LCR is difficult to explain. It may be that a regulatory sequence like an insulator, a cis-acting element that shields the transcriptional units from the surrounding DNA, exists in the full-length LCR but not in HS core elements. In fact, LCR containing HS4, 3, 2, 1 does not confer position-independent expression onto the b-globin promoter-driven lac Z gene in transgenic mice (Guy et al. 1996; Guy et al. 1997). HS5 59 to HS4 was shown to have the properties of an insulator (Li and Stamatoyannopoulos 1994). At present, the site of integration usually cannot be chosen or controlled because viral vectors integrate at random into the chromosomes of target cells. An insulator is recommended to elude position-effect after integration by viral vectors. A number of insulators have been detected in Drosophila (Kellum and Schedl 1991; Kellum and Schedl 1992; Holdridge and Dorsett 1991; Geyer and Corces 1992; Roseman et al. 1993; Cai and Levine 1995; Scott and Geyer 1995), chickens (Chung et al. 1993; Bonifer et al. 1994), and humans (Li and Stamatoyannopoulos 1994; Kalos and Fournier 1995). A study of the chicken b-globin locus suggested that the chromatin near the 59 constitutive DNase I-hypersensitive site was condensed (Reitman and Felsenfeld 1990). It has subsequently become apparent that a sequence at the 59 end of the chicken b-globin locus, containing a constitutive DNase I-hypersensitive site, can function as an insulator (Chung et al. 1993). Most of the insulating activity lies in a 250-bp core element, which contains the constitutive DNase I-hypersensitive site (Chung et al. 1997).

We expected that an rAAV vector with an insulator would be capable of reducing the variability of expression of transduced genes. One limitation of the AAV system for gene therapy is that the virion can package DNA only up to

5.0 kb (Berns and Bohenzky 1987). In this study, a 250-bp fragment was ligated in tandem and inserted in both sides of rHS2 because of size limitation for efficient packaging of the viral genome. We demonstrated that the minimal insulator comprising juxtaposed tandem copies of the 250-bp core sequence provided insulator function in MEL cells infected by the rAAV vector. These findings indicate that this minimal insulator would exert effects on chromatin structure and overcome position effect variegation in gene therapy. How are insulator sequences capable of preventing position effects on the transcription unit and do they block the effects of regulatory sequences on neighboring sequences at the site of integration? They may act either by preventing tracking of a distant enhancer complex along chromatin or by blocking access to any factors outside the loop formed by the interaction of the 5′ insulator with the 3′ insulator (Chung et al. 1993; Corces 1995). Further, it is also noteworthy that a 250-bp element used in this study has the properties of a CpG island (Chung et al. 1997). Mechanisms of the insulator function remain unknown.

Most current gene therapy approaches make use of viral vectors (Verma and Somia 1997). This is a powerful technique, as specific machinery has evolved to deliver genes to cells. In recent years, the AAV vector has received increased attention because of its potential as a vector for gene therapy, and recent studies have shown that the AAV vector could be used to introduce specific genetic changes into the genomic DNA of mammalian cells, including a therapeutic gene targeting application (Russell and Hirata 1998). AAV is non-pathogenic (Berns and Bohenzky 1987), resistant to chemical and physical treatments (Berns and Bohenzky 1987), and infects various types of tissues, such as brain (McCown et al. 1996), liver (Snyder et al. 1997a), retina (Ali et al. 1996; Ali et al. 1998), and skeletal muscle (Fisher et al. 1997; Snyder et al. 1997b). However, a study using immortalized epithelial cells indicated that the rAAV vector integrated at a low frequency and persisted as an episome in infected cells (Kearns et al. 1996). Recently, less efficient integration of the rAAV in the human erythropoietic cell line, K562, was reported (Malik et al. 1997), indicating that AAV is not necessarily an appropriate system for gene transfer to human hematopoietic progenitor cells. We observed that a single rAAV vector genome integrated at random in host chromosomes on target cells, as determined by Southern blot analysis of genomic DNA and FISH analysis in this rAAV-MEL cell system. Long-term expression of the human globin gene was observed in bone marrow of recipient mice reconstituted by low-density bone marrow mononuclear cells infected with an rAAV carrying the human globin gene (Ponnazhagan et al. 1997), suggesting the potential usefulness of the AAV-based vector system in gene therapy for human hemoglobinopathies. For gene therapy, expression levels of the transduced gene must persist long-term. We are currently investigating the effects of the insulator on the stability of the vector genome and expression levels of the transduced gene in continuous long-term culture.

As this insulator protects the *white* minigene from position effects in transgenic *Drosophila* and as AAV has a wide range regarding susceptible cell type, rAAV with the insulator could be an effective gene delivery system to provide consistent expression of non-erythroid genes in target tissues, in addition to the globin gene reported here. This system not only decreases the variability of expression of a transduced gene but also avoids activation of an oncogene near the insertion site of the recombinant viral genome.

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