Retroviral transfer of a human β -globin/ δ -globin hybrid gene linked to β locus control region hypersensitive site 2 aimed at the gene therapy of sickle cell disease

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ABSTRACT Human γ -globin and δ -globin chains have been previously identified as strong inhibitors of the polymerization of hemoglobin S, in contrast to the β -globin chain, which exerts only a moderate antisickling effect. However, γ -globin and δ -globin are normally expressed at very low levels in adult erythroid cells, in contrast to β -globin. We report the design of a β -globin/ δ -globin hybrid gene, β / δ -sickle cell inhibitor 1 (β / δ -SCI1) and its transduction by retrovirus-mediated gene transfer. The β / δ -SCI1-encoding gene retains the overall structure of the human β -globin gene, while incorporating specific amino acid residues from the δ chain previously found responsible for its enhanced antisickling properties. To achieve high expression levels of β / δ -SCI1 in adult erythrocytes, the hybrid gene was placed under the transcriptional control of the human β -globin promoter and the DNase I hypersensitive site 2 of the human β locus control region. High-titer retroviruses were generated, and stable proviral transmission was achieved in infected cells. The mRNA expression levels of the β / δ -SCI1 gene in infected, dimethyl sulfoxide-induced murine erythroleukemia cells approached 85% of the endogenous murine β_{maj} -globin mRNA, on a per gene basis, evidence that high gene expression levels were achieved in adult erythroid cells. Further evaluation of this strategy in transgenic animal models of sickle cell disease should assess its efficacy for the gene therapy of human patients.

The sickling of erythrocytes in sickle cell disease (SCD) is the result of a single amino acid mutation, β -chain (Glu-6 $\rightarrow$ Val), which leads to the polymerization of hemoglobin S ($\alpha_2\beta_2^S$) in the tense (T), deoxygenated state. Long, multistranded fibers form within erythrocytes of patients with SCD. The main fibers are made of 14 twisted strands associated by pairs (the Whisher–Lowe double strand). The β -chain Val-6 mutation is required to initiate stable lateral contacts with another tetramer in the double-stranded unit. Only one β -chain Val-6 mutation per $\alpha_2\beta_2^S$ tetramer is involved in intertetramer contact, although many other amino acid residues participate in lateral and axial contacts within the double strand as well as between double strands (for review, see refs. 1–4).

Mixing β^S with the other normal human β -like globin chains (β^*)—i.e., β -, δ -, or γ -, results in an equilibrium between symmetrical ($\alpha_2\beta_2^S$ and $\alpha_2\beta_2^*$) and hybrid $\alpha_2\beta^S\beta^*$ tetramers because interactions at α/β subunit interfaces within the same hemoglobin tetramer (packing and lateral contacts) are similar among β -like globin chains. Symmetrical tetramers that do not have the β -chain Val-6 mutation ($\alpha_2\beta_2^*$) are incorporated into the fibers very poorly. The $\alpha_2\beta^S\beta$ hybrid tetramers are capable of copolymerizing with hemoglobin S because only one β -chain Val-6 residue per tetramer is required for intertetramer con-

tact, and the other lateral and axial contacts are formed efficiently with the trans β subunit. In contrast, $\alpha_2\beta^S\gamma$ and $\alpha_2\beta^S\delta$ hybrid tetramers are poorly, if at all, incorporated because trans δ - and γ -globin chains are unable to form important contacts within the S fiber, even when the β -chain Val-6 residue of the β^S subunit is aligned appropriately. This phenomenon is believed to explain why γ - and δ -globins are much stronger inhibitors of sickling than β -globin, *in vitro*. In addition, interaction of these hybrid tetramers with hemoglobin S without successful copolymerization is expected to delay the polymerization process *in vivo*, so that erythrocytes return to the lung and are reoxygenated before significant sickling has occurred (refs. 5–7; for review, see refs. 1–4).

The actual inhibitory effect of δ chains has not yet been assessed *in vivo*, because the δ -globin gene is expressed at very low levels in human erythrocytes. In contrast, there is a strong correlation between high expression levels of γ chains and a lower propensity for sickling *in vivo*, as observed for instance in certain forms of SCD associated with hereditary persistence of fetal hemoglobin (for review, see refs. 1–4). In addition, drugs known to derepress partially γ -globin gene expression, such as hydroxyurea and butyrate derivatives, are beneficial to SCD patients. However, these approaches do not represent a definitive cure and have raised legitimate concerns regarding their potential long-term side effects. In addition, drug-induced γ -globin expression might be largely restricted to F cells, so that non-F cells may still sickle in SCD (for review, see ref. 8).

At present, allogeneic bone marrow transplantation with HLA-matched hematopoietic stem cells from normal individuals remains the only available possibility of cure for patients with SCD. However, most do not have an HLA-matched donor, and the procedure is associated with a significant risk of mortality and morbidity. As an alternative, retroviral transfer into hematopoietic stem cells of a gene construct resulting in high and erythroid-specific expression of a β -like globin chain having strong antisickling properties is an attractive approach for the gene therapy of SCD. The discovery of the β locus control region of the human β -globin gene cluster (β -LCR) (9–11) and its detailed characterization (for review, see ref. 8) have given promise of achieving high and steady expression of therapeutic genes in erythroid cells. Although the design of retroviral vectors transducing β -globin gene and β -LCR derivatives has been faced with important technical difficulties (12–14), we have recently obtained (β -globin/LCR) retroviral vectors capable of stable proviral transmission and high erythroid-specific β -globin mRNA expression (15).

Abbreviations: C/R, complementary/reverse; DMSO, dimethyl sulfoxide; HS, DNase I hypersensitive site; IVS, intervening sequence or intron; β -LCR, β locus control region of the human β -globin gene cluster; MEL, murine erythroleukemia; SCD, sickle cell disease; β / δ -SCI1, β / δ -sickle cell inhibitor 1; SS, splice site(s); PGK, murine phosphoglycerate kinase 1.

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Because the β chain has only moderate antisickling properties and because γ - and δ -globin genes are normally expressed at very low levels in adult erythrocytes, we have designed a β/δ -globin gene hybrid, referred to as β/δ -sickle cell inhibitor 1 (β/δ -SCI1), by substituting nucleotides encoding "antisickling" amino acid residues from the δ chain (16) for corresponding codons of the β -globin gene in a [β -globin/hypersensitive site (HS)2] retroviral vector. Proviral transmission and expression properties of this (β/δ -SCI1/HS2) retroviral vector, p#147, were subsequently analyzed.

MATERIALS AND METHODS

DNA Construction. The retroviral vector (β -globin/LCR/murine phosphoglycerate kinase 1 (PGK)^{mut} has already been described (15) and derives from LXSNI(17). The δ -globin gene was provided by Oliver Smithies (University of North Carolina, Chapel Hill). The β/δ -SCI1 gene and the retroviral vector p#147 were constructed as described in *Results*. Constructs used to generate probes for RNA protection assays are described below. Constructions were made by using standard techniques (18). Oligonucleotides were synthesized at the Massachusetts Institute of Technology (MIT) Biopolymer Laboratory.

Production of Recombinant Retroviruses. The packaging cell lines Ψ cre and Ψ crip, provided by Richard Mulligan (Whitehead Institute and MIT, Cambridge, MA), were grown, transfected, and G418-selected, as described (19). The packaging cell line BOSC23, provided by David Pear and David Baltimore (Rockefeller University, New York), was grown and transfected, as described (20). Because p#147 is a self-inactivating retroviral vector, plasmid DNA was directly transfected into packaging cells using a calcium phosphate procedure (5 Prime $\rightarrow$ 3 Prime, Inc.), after linearization of the plasmids outside the proviral structure (*Nde* I site) for Ψ cre and Ψ crip cells, or without linearization for BOSC23 cells. Viral supernatants from either stable Ψ cre and Ψ crip producers or transiently transfected BOSC23 cells were harvested as described (refs. 19 and 20, respectively). Detection of helper viruses was performed by a β -galactosidase mobilization assay as described (19).

Proviral Transmission and Virus Titration. NIH 3T3 cells were infected with various dilutions of filtered viral supernatants and subsequently G418-selected, as described (15, 19). Resistant colonies were counted, and titers were estimated by standard calculations as described (15, 19). Proviral transmission was tested by Southern blot analysis of *Sac* I-digested DNA from infected cells using a neo^R-specific probe and appropriate plasmid controls.

Infection of Murine Erythroleukemia (MEL) Cells and RNA Protection Assays. Semiadherent MEL cells were grown, infected, G418-selected, and induced with dimethyl sulfoxide (DMSO), as described. Total RNA was extracted by the RNazol B method (Biotecx Laboratories, Houston). Quantitative RNA protection assays were done with uniformly labeled RNA probes, *in vitro* transcribed using SP6 (GIBCO) (for the β_{maj} probe) or T7 polymerase (GIBCO) (for the δ probe) in the presence of [α -³²P]UTP (Amersham), under the same experimental conditions as described (15), except that the samples were combined at 1:2 ratio ($\beta_{maj}:\delta$) before loading onto a 6% polyacrylamide gel, as described in Fig. 5. A human δ -globin-specific probe was constructed by subcloning an oligonucleotide containing a T7 promoter in reverse genomic orientation at the *Bam*HI site located in the second exon of the human δ -globin gene, and linearizing the template before *in vitro* transcription at the *Nco* I site in δ -globin exon I. The main protected fragment, upon hybridization to the human δ -globin mRNA, is 210 bp long and corresponds to most of δ -globin exon II up to the exonic *Bam*HI site. A described murine β_{maj} -globin-specific probe (15), was used as an internal control

for murine endogenous mRNA expression. As reported (15), this probe protects a 145-bp fragment corresponding to exon I of the murine β_{maj} -globin mRNA, as well as a 115-bp fragment of the murine β_{min} -globin mRNA, in a region of high sequence homology with the β_{maj} probe. Radioactive bands corresponding to the specific protected fragments were scanned by using a PhosphorImager (Molecular Dynamics). The mRNA ratios were calculated on a per gene basis in MEL cells, which are pseudodiploid for the endogenous murine globin genes (correction factor, 2). The mRNA ratio was also corrected for the number of uridine residues in each protected fragment of the probes (44 for human δ and 33 for murine β_{maj} ; correction factor, 1.3), and the 1:2 ratio ($\beta_{maj}:\delta$) before loading (correction factor, 2). Global calculations were made as follows:

$$\frac{\text{human } \delta}{\text{murine } \beta_{maj}} = \frac{[\text{human } \delta\text{-globin band}] \times 2 \times 100}{[\text{murine } \beta_{maj}\text{-globin band}] \times 2 \times 1.3}$$

RESULTS

Rationale and Design of β/δ -SCI1 Gene and Retroviral Vector p#147. Because of the few differences between β and δ chains (10 of their 146 residues), Nagel *et al.* (16) have succeeded in assigning most of the *in vitro* antisickling properties of δ chains to residues δ -chain Ala-22 and Gln-87, with a possible additional contribution of δ -chain Asn-12. Recently, Nagel's findings were confirmed by showing that a recombinant hemoglobin that contains δ -chain Ala-22 and Gln-87 inhibits efficiently hemoglobin S polymerization *in vitro* (21). In addition, additional residues of the δ chain quite probably cooperate with these two main amino acid substitutions for the complete antisickling effect of the δ chain, by interfering with other axial, lateral intrafiber or interfiber contacts (refs. 3 and 22; Figs. 1 and 2). In contrast, the definition of the critical antisickling residues of γ chain was less unequivocal due to the large number of differences between γ and β chains (39 of their 146 residues) (16), besides γ -chain Gln-87, which is identical to δ -chain Gln-87.

The study of Lepore β/δ -hybrid globin genes has also provided insights into the differential expression properties of cis-acting elements proximal to the δ - and β -globin genes: the β -globin promoter and the second intron of the β -globin gene (β IVS2, where IVS represents intervening sequence or intron) appear critical for high mRNA expression in adult erythroid cells (23). Interestingly, δ -chain Ala-22 and δ -chain Gln-87 are encoded by δ -chain exons I and II. In an effort to obtain high expression levels in adult erythroid cells of a β -like globin chain with antisickling properties, we chose, therefore, to design a hybrid β/δ -globin gene, β/δ -SCI1, that consists of the following: (i) the β -globin promoter linked to the (*Hind*III-*Xba* I) HS2 fragment of the human β -LCR (24-27), in direct genomic orientation, (ii) the 5' untranslated region of β exon I, (iii) the coding region of δ exon I, δ IVS1 and most of δ exon II, (iv) exon III of the β -globin gene with β IVS2, and (v) a short flanking region 3' of the β -globin gene. As mentioned above, exons I and II of the δ -globin gene encode all the residues believed to contribute to the antisickling activity of the δ chains. Because changes in the charge of globin chains are known to interfere with tetramer assembly or stability, especially with positively charged mutants (for review, see refs. 1 and 3), we also chose to substitute codons 116 and 117 of the δ chain for corresponding codons in exon III of the β -globin gene: (β -chain His-116 to δ -chain Asn-116) removes a positive charge to compensate for the loss of negative charge resulting from (β -chain Glu-22 to δ -chain Ala-22) (Fig. 1).

The β/δ -SCI1 gene was constructed directly in the retroviral vector (β -globin/HS2/PGK)^{mut} that we have described (15), to yield a (β/δ SCI1/HS2) retroviral vector referred to as p#147.

To study proviral transmission and to measure viral titers, MEL and NIH 3T3 cells were infected by exposure to viral supernatants from pooled Ψ crip stable producer cells (Fig. 4) or from transiently transfected BOSC23 cells. Infected cells were subsequently selected with G418. Proviral transmission was analyzed by Southern blot using a neo^R-specific probe after restriction digestion of genomic DNA with *Sac* I, which cuts only once in each long terminal repeat. Corresponding digested plasmids were used as size controls. Results from the Southern blot analysis indicate that proviral transmission was stable, without detected rearrangements. Viral titers, obtained from transiently transfected BOSC23 cells, were 2×10^4 colony forming units/ml for both the retroviral vector p#147 and (β -globin/HS2/PGK)^{mut}, an indication that these vectors had very similar transmission properties. In light of our previous results with (β -globin/HS2/PGK)^{mut} producers (15), we expect the best independent p#147 Ψ cre producers to reach titers $>10^5$ colony-forming units/ml.

No helper virus was detected by using a β -galactosidase mobilization assay in any of the viral producers.

Expression Properties of (β / δ -SCI1/HS2) Retroviral Vector p#147. The main goal of this work was to generate retroviral vectors conferring high expression levels of the hybrid β / δ -SCI1 gene in adult erythroid cells. Toward this aim, we have examined β / δ -SCI1 mRNA expression levels in DMSO-induced MEL cells, which mimic adult erythroid differentiation. MEL cells were infected with supernatants from amphotropic producers under experimental conditions that provided up to one integrated provirus per cell and were subsequently selected with G418. Pools of at least 100 infected and selected MEL cell clones were analyzed, either without induction or after exposure to DMSO for 5 days to induce terminal erythroid differentiation. Human and murine β -globin mRNA levels were measured by RNA protection assay by using probes specific for DNA sequences in either murine β_{maj} - or human δ -globin mRNAs, as described. Our results (Fig. 5) demonstrate that human β / δ -SCI1 mRNA was properly spliced in infected MEL cells. The mRNA ratios were calculated on a per gene basis, with appropriate corrections (see *Materials and Methods* for calculations). In infected (one provirus per cell), G418-selected, and DMSO-induced MEL cells, expression levels of β / δ -SCI1 mRNA using the p#147 virus reached 85% of murine β_{maj} -globin mRNA, corrected on a per-gene basis as described.

DISCUSSION

This study was aimed at assessing the feasibility of achieving efficient retrovirus-mediated transfer of an "antisickling"

FIG. 4. Southern blot analysis of proviral transmission of (β / δ -SCI1/HS2) retroviral vector p#147. Each lane contains *Sac* I-digested genomic DNA from a pool of MEL cells infected from a pool of Ψ crip producers. Lanes: 1, infection with the p#147 virus; 2, infection with the (β -globin/HS2/PGK)^{mut} virus, as size control for stable proviral transmission.

FIG. 5. RNA protection assays. MEL cells were infected with amphotropic p#147 virus obtained from a pool of Ψ crip producers, under conditions providing up to one provirus per cell. Infected MEL cells were subsequently selected with G418 and pooled. Total RNA was extracted from pools (>100 independent clones) of infected and G418-selected MEL cells, either without induction or after induction with DMSO for 5 days (two independent experiments of DMSO induction were done). For each RNA sample from p#147-infected cells, RNA protection assays were done separately with the same RNA amount, using the murine β_{maj} -globin-specific probe or the human δ -globin-specific probe, as described. The digested samples were subsequently combined at a 1:2 ratio (β_{maj} : δ) before loading onto a 6% polyacrylamide gel, so that each of lanes 2–4 contains protected bands obtained with both murine β_{maj} -globin and human δ -globin probes. Positions of undigested probes are indicated by asterisks. Positions of specific protected fragments are indicated, as follows: H δ , human δ chain; M β_{maj} , murine β_{maj} chain; M β_{min} , murine β_{min} chain. Lanes: 1, noninfected, DMSO-induced MEL cells, with the murine β_{maj} -globin probe alone; 2, p#147 infected, G418-selected, noninduced MEL cell pool; 3 and 4, p#147-infected, G418-selected, DMSO-induced MEL cell pools, corresponding to two independent DMSO inductions.

globin gene that is expressed at high levels in adult erythroid cells. For reasons given above, our first choice of strategy focused on the antisickling amino acid residues of the human δ -globin chain. Toward this goal, a hybrid δ -globin/ β -globin gene, β / δ -SCI1, was constructed. This gene construct contains the cis-acting elements responsible for high expression of the human β -globin gene in adult erythroid cells, (i.e., β -globin promoter and β IVS2) but encodes a complete human δ -globin chain, with the exception of two amino acid residues from the human β -globin chain. In particular, β / δ -SCI1 encodes all the amino acid residues previously identified as conferring the strong antisickling properties of the human δ -globin chain (16, 21). In addition, the β / δ -SCI1 gene, driven by the β -globin promoter, was linked to the HS2 enhancer from the human β -LCR. Our results indicate that recombinant retroviruses with high viral titers compatible with stable proviral transmission were generated and that high mRNA expression levels in cell lines mimicking adult erythroid differentiation were obtained.

Instability of proviral transmission has represented a major technical hurdle to the design of retroviral vectors transducing β -globin gene and β -LCR derivatives (13, 14, 16). We have recently reported that proviral transmission was rendered stable by extensive mutagenesis of (β -globin/LCR) retroviral vectors (15). This mutagenesis removed a 372-bp fragment of β IVS2 and most of the 3' flanking region of the gene, and eliminated cryptic C/R SS and poly(A) from the β -globin gene (15). In the present study with p#147, the 372-bp deletion in β IVS2 and several mutated cryptic SS in exon III of the β -globin gene were maintained; however, the potentially dangerous C/R SS within the *Nco* I–*Bam*HI fragment of the δ -globin gene portion of p#147 were not removed. Despite these cryptic SS, p#147 viruses appeared stable, and viral titers were similar to those obtained with the parent vector (β -

globin/HS2/PGK)^{mut}. It remains possible that, if additional LCR derivatives are added to this construct to increase β/δ -SC11 mRNA expression, these cryptic SS might have to be removed for viral transmission to remain stable.

Our results indicate that our aim of driving the expression of a β/δ -hybrid globin gene in adult erythroid cells was achieved. The mRNA expression levels in DMSO-induced MEL cells were found to be slightly higher for p#147 than for (β -globin/HS2/PGK)^{mut}, $\approx 85\%$ versus 70% of endogenous β_{maj} -globin expression, respectively (15). It is somewhat surprising that the level of gene expression measured for the p#147 vector was higher than that obtained with the parental β -globin retroviral vector. The reason for this perceived difference in mRNA expression levels is unknown. Besides HS2, it might be necessary to incorporate additional β -LCR derivatives to boost β/δ -SC11 gene expression further *in vivo*, with relative independence from the sites of chromosomal integration at single proviral copy.

Other strategies of design of antisickling genes that might be expressed at high levels in adult erythrocytes could include the use of a γ -globin gene driven by LCR derivatives linked to either a β -globin promoter or a γ -promoter that contain mutations described in hereditary persistence of fetal hemoglobin syndromes.

As a complement to the present study focused on mRNA expression, characterization of the β/δ -SC11 protein made *in vivo* will have to be done, with regard to protein translation, stability, and hemoglobin tetramer assembly and function. The antisickling properties of β/δ -SC11 should also be assessed in transgenic animal models of SCD (for review, see ref. 28).

Before SCD becomes amenable to gene therapy in human patients, significant improvements of gene-transfer efficiencies and bone marrow transplantation protocols should also be obtained to approach the ultimate goal of complete and sustained reconstitution with only transduced hematopoietic stem cells in all transplanted individuals.

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