

Multi-ribozyme Targeting of Human α -Globin Gene Expression

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ABSTRACT: One approach to gene therapy for the treatment of hemoglobinopathies has been focused on increasing normal globin gene expression. However, because of the high concentration of hemoglobin in the red blood cell (32–34 g/dl), merely introducing the normal globin gene may not be enough to counteract the effect of an abnormal globin. We propose that in addition to strategies to add normal β - or γ -globin production to sickle erythrocytes, a decrease in overall hemoglobin concentration would further decrease the polymerization potential and should be considered with other gene therapy approaches. Ribozymes offer the potential to target a selected gene product. A model system has been set up using the human α -globin gene for specific gene suppression by ribozymes by cleaving α -globin mRNA transcripts. Ribozymes, specifically targeted to five different sites in the 5' portion of human α -globin mRNA, have been designed and tested *in vitro*. Cleavage of ³²P-labeled α -globin mRNA by these ribozymes has been observed *in vitro* and the highest level of activity has been found for a multi-ribozyme combining all five ribozymes. The multi-ribozyme gene along with promoters with varying activities in erythroid cells was transfected into human erythroleukemia K562 cells. The multi-ribozyme gene, under the control of human α -2-globin promoter alone and combined with the locus control region enhancer, caused a decrease in the level of α -globin mRNA of 50–75% compared to the control, determined by RNase protection and by real-time quantitative PCR. The decrease in α -globin transcripts has been found to be correlated with expression of the multi-ribozyme in a dose-dependent manner and does not appear to be mediated by an antisense effect. These results suggest that the multi-ribozyme may be useful in gene therapy as an effective suppressor of a specific globin gene.

Keywords: ribozyme, multi-ribozyme, gene therapy, α -globin mRNA, K562 cells

INTRODUCTION

Almost 700 naturally occurring mutations in human α - and β -globin genes have been identified and about 140 of them have been shown to induce serious blood diseases (1,2). Gene therapy may be a promising method for treatment of hemoglobinopathies. Efforts have been made to introduce supplemental expression of the normal β - or γ -globin gene (3–5) and to track (6) and maintain prolonged expression of the transgene (7–9). However, unlike other gene-deficiency diseases that may be cured by introducing the normal gene into proper target cells, for most hemoglobin (Hb)-based blood diseases, because of the high concentration of Hb in the red blood cell (32–34 g/dl), merely introducing the normal globin gene may not be enough to counteract the effect of an abnormal globin gene. In the case of sickle cell anemia, the non-ideal behavior of concentrated protein solutions results in a strong concentration dependence of the polymerization

of sickle cell hemoglobin [Hb S (β 6Glu \rightarrow Val)]. Addition of protein, such as normal Hb, that results in an increase in total protein concentration can adversely affect the polymerization potential and can increase the extent of Hb S polymerization (10,11). Even if the expression of the introduced normal globin gene is very high, the symptoms may still exist depending on the resultant high Hb concentration and increased amount of Hb produced in the red blood cell. For example, in Hb SC disease [individuals heterozygous for Hb S and Hb C (β 6Glu \rightarrow Lys)], the SC erythrocyte contains 50% Hb S with an increased total Hb concentration. Although the difference in proportion of Hb S between benign sickle trait (AS) and Hb SC disease is only 10%, when combined with the increase in Hb concentration, there is a significant increase in the polymerization potential associated with SC disease. Addition of normal β -globin production without decreasing the mutant β^S -

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globin production could increase the overall Hb concentration and further increase the polymerization potential in sickle red blood cells (11). Therefore, for gene therapy of blood diseases, effective suppression of the endogenous α - or β -globin gene in the blood cells to maintain or reduce the total Hb concentration may be as important as the introduction of normal globin genes into the blood cells.

Ribozymes have been widely used to suppress the mRNA or viral RNA of interest at the transcriptional level. A number of ribozymes have been designed and tested in cell lines targeting cellular and viral genes (12-14), their targets include infection by HIV (15) and

hepatitis C (16), oncogene production (17) and blocking metastasis (18) or abrogating apoptosis (19). Recently, a single ribozyme has been designed to target β^S -globin mRNA and introduced into transgenic mice (20). However, in this case, only limited reduction of β^S -globin mRNA level was observed.

Here, we propose a strategy employing multi-ribozyme to reduce the production of the abnormal globin gene product. In this study, we have set up a model system using the human α -globin gene as the target for suppression by gene-specific ribozymes. Five ribozymes have been designed to target specific sites on the

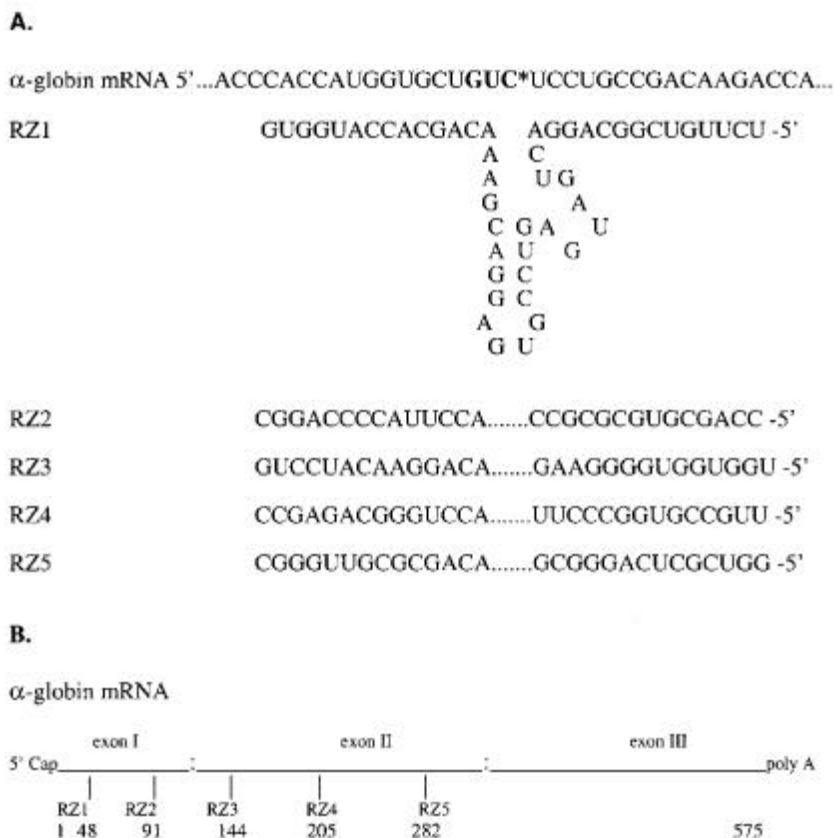


Figure 1. Ribozymes designed against human α -globin mRNA: A, The total sequence of RZ1 and its corresponding target α -globin mRNA fragment are shown at the top. The ribozyme target sequence is in bold type. The cleavage site is labeled with *. For the ribozymes RZ2-5, only the sequences of the arms are shown. B, The approximate locations of the cleavage sites of RZ1-RZ5 on human α -globin mRNA are indicated.

human α -globin mRNA. A multi-ribozyme combining all five ribozymes has also been synthesized. In a cell-free system, the ribozyme constructs are able to specifically cleave α -globin mRNA, with the multi-ribozyme combination being the most effective. Introduction of the multi-ribozyme construct into human erythroleukemia K562 cells results in a significant and dose-dependent reduction in the intracellular level of α -globin mRNA. Such a procedure should be useful in both suppressing the expression of abnormal globin gene and regulating

globin chain balance when combined with targeted increased expression of a normal globin gene.

MATERIALS AND METHODS

Ribozyme Design and Oligonucleotide Synthesis

Five hammerhead ribozymes, RZ1-RZ5, against the 5'-portion of human α -globin mRNA have been designed. The ribozymes are 50

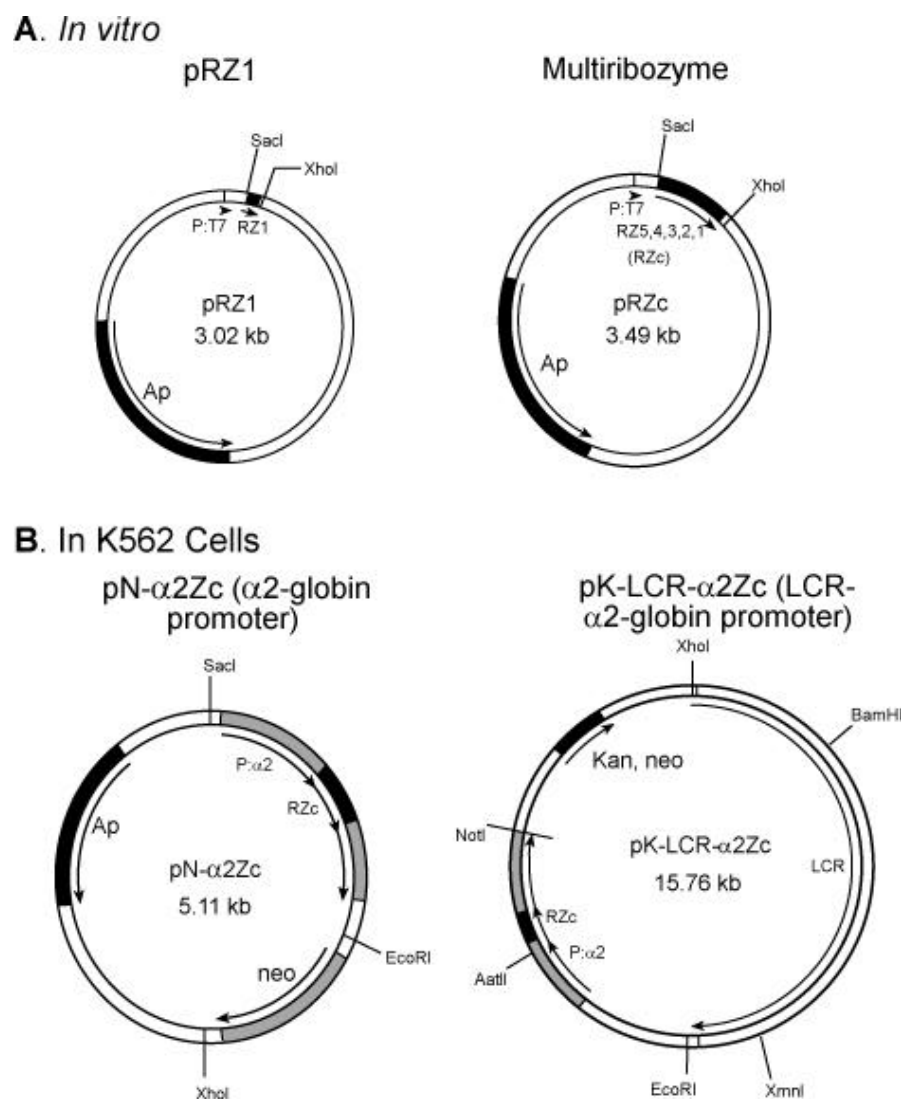


Figure 2. Plasmids for producing ribozymes used *in vitro* (A) and in K562 cells (B). The construction of the plasmids is described in Materials and Methods. P: T7, P: α -2 designate the promoter fragments for T7 and α -2-globin, respectively. LCR designates the HS4, HS3, HS2 and HS1 sequences of the human β -globin gene cluster. RZc or Zc designates the penta-ribozyme gene. Plasmids pRZ2-5 are constructed in the same manner as pRZ1.

nucleotides long with each arm having 14 their presumed cleavage sites on human α -globin mRNA are shown in Figure 1. The corresponding oligodeoxyribonucleotides of these ribozymes were synthesized as sense and antisense oligonucleotides (Applied Biosystems 380B DNA Synthesizer, Foster City, CA) and annealed for ligation into plasmids.

Construction of Plasmids for Producing Single and Multi-ribozymes Used in Vitro and in K562 Cells

The five synthetic ribozyme genes were inserted into the Sma I site of plasmid pBluescript II (Stratagene, La Jolla, CA) to form plasmids pRZ1-5 (Figure 2A). For the construction of the penta-ribozyme gene RZc, a 1.5-kb PstI genomic fragment containing the human α -2-globin gene (GenBank Accession No. J00156, 6092-7586) was inserted into plasmid pBluescript II to form plasmid pKSh α 2g. A Nsi I site was introduced into intron I of the α -2-globin gene in pKSh α 2g to form plasmid pKSh α 2gNsi. Three site-directed mutagenesis procedures were performed with the Gene Editor Mutagenesis System (Promega, Madison, WI) using pKSh α 2gNsi as template and oligodeoxyribonucleotides corresponding to RZ1 and RZ2, RZ3 and RZ4, and RZ5 as mutated primers. Thus, the ribozyme sequences RZ1 and RZ2, RZ3 and RZ4, and RZ5 were respectively introduced into the resulting three mutated plasmids pKS α 2-RZ1,2, pKS α 2-RZ3,4, and pKS α 2-RZ5. The 1.17-kb BsaA I fragment of pKS α 2-RZ5 and the 3.35-kb BsaA I fragment of pKS α 2-RZ3,4 were ligated to form pKS α 2-RZ3,4,5 which contains the RZ3, RZ4, and RZ5 sequences. The 3.8-kb Nsi I-EcoR I fragment of pKS α 2-RZ3,4,5 and the 0.75-kb Nsi I-EcoR I fragment of pKS α 2-RZ1,2 were ligated to form pK α 2-RZ1,2,3,4,5, which contains all five ribozyme gene sequences. Finally, the oppositely oriented 0.53-kb Nco I-Tth111 I fragment of pKS α 2-RZ1,2,3,4,5 was inserted into the Sma I site of pBluescript II. The resultant plasmid pRZc contains all five ribozyme genes arranged in RZ5,

nucleotides. The structure of these ribozymes and RZ4, RZ3, RZ2, and RZ1 order, and the spacing sequences between them are exclusively antisense α -2-globin gene sequences (exon II, I and intron I). In plasmids pRZ1-5 and pRZc, all the ribozyme genes were placed behind the T7 promoter and used for producing single and multi-ribozyme RNAs with the *in vitro* T7 transcription system (Promega).

For K562 cell transfection experiments, the coding sequence of the α -globin gene in pKSh α 2g was deleted by digestion with Nco I and Apa I and the penta-ribozyme gene RZc (oppositely oriented 0.53-kb Nco I-Tth111 I fragment from pKS α 2-RZ1,2,3,4,5) was inserted into the deleted vector to form plasmid pKS α 2Zc. This plasmid was then fused with plasmid pMC1polyA (Stratagene) to form plasmid pN- α 2Zc which contains both the neomycin resistance marker from pMC1polyA and the penta-ribozyme gene RZc from pKS α 2Zc under the control of the human α -2-globin promoter (Figure 2B). Plasmid pN-SVZc was similarly constructed, replacing the α -2-globin promoter with the SV40 early promoter (Promega). Plasmid pN- α 2As was also constructed in the same manner as pN- α 2Zc, except the multi-ribozyme gene RZc was replaced by an oppositely oriented Nco I-Tth111 I fragment of pKSh α 2g which contains antisense exons II, I and intron I sequences of the human α -2-globin gene. Another multi-ribozyme expression plasmid pK-LCR α 2Zc was constructed by placing the locus control region (LCR) enhancer sequence ahead of the α -2-globin promoter of pKS α 2Zc, and fusing the resulting plasmid with a kanamycin-neomycin resistance marker from plasmid pEGFP-1 (Clontech, Palo Alto, CA). A control plasmid pK-LCR- α 2 Δ NT was similar to pK-LCR- α 2Zc except the Nco I-Tth111 I multi-ribozyme fragment was completely deleted. The LCR enhancer used in these plasmids contains the Sac I-Sac I fragment of HS4, the BamH I-HindIII I fragment of HS3, the Kpn I - Bgl II fragment of HS2, and the Sac I-EcoRI fragment of HS1

(GenBank Accession No. J00179, –18589 - –14365, –15664 - –14364, –11777 - –10308 and –6794 - –2064). Plasmids pN- α 2Zc, pN-SVZc, pN- α 2As, pK-LCR- α 2Zc, and pK-LCR- α 2 Δ NT were used in the experiments in K562 cells.

In Vitro Ribozyme Cleavage Assay

Plasmid pKSh α 2g which contains the human α -2-globin gene (see above), was digested with Eag I. The 3.94-kb fragment was self-ligated to generate plasmid pKSh α 2g Δ 5', in which the α -2-globin promoter is deleted and the transcribed portion of the α -2-globin gene is placed behind the T7 promoter. The 0.32-kb Nco I-BstE II fragment of human α -globin cDNA (from plasmid p α c, provided by Dr. Susan J. Baserga and Dr. Maureen Gilmore-Herbert) was inserted into the 3.36-kb fragment of Nco I- and BstE II-digested pKSh α 2g Δ 5' to obtain plasmid pKSh α c', in which the human α -globin cDNA sequence is placed behind the T7 promoter. There is an additional 83 bp between the T7 promoter and the human α -globin sequence in both plasmids pKSh α c' and pKSh α 2g Δ 5'.

With Spe I-linearized pKSh α c' and Xho I linearized pKSh α 2g Δ 5' as templates, 32 P-labeled human α -globin mRNA (coding sequence) and 32 P-labeled human α -globin pre-mRNA (including introns I and II) were synthesized using a T7 RNA polymerase *in vitro* transcription system (RiboMAX kit, Promega). With Xho I-linearized plasmids pRZ1-5 and pRZc as templates, unlabeled ribozyme RNAs RZ1-5 and RZc were also synthesized by *in vitro* transcription. The synthesis mixtures (total volume 40 μ l) contained 4 μ g of linearized plasmid DNA, 8 μ l of transcription buffer, 10 mM of each of four NTP, 2 μ l of [α - 32 P] UTP (800 Ci/mmol, Amersham, Arlington Heights, IL, added only when labeled RNA was needed), and 4 μ l of enzyme mix (300 u/ μ l T7 RNA polymerase, 15 u/ μ l RNasin and 190 u/ μ l yeast inorganic pyrophosphatase), and were incubated at 37 C for 2 h. Then, 2 μ l of RNase-free DNase (2 u/ μ l) was added and the

mixtures were incubated at 37 C for another 30 min. After that, the reaction mixtures were passed through CHROMA SPIN-30 + DEPC-H₂O Columns (Clontech). The concentration of the labeled α -globin mRNA or pre-mRNA was estimated by the total isotope incorporation in the synthesis reaction, the concentration of synthesized ribozyme RNAs was checked by UV-absorption, and both were adjusted to 0.2 μ M by adding DEPC-treated water.

The *in vitro* ribozyme cleavage reaction was carried out as follows: The reaction mixtures contained 1 μ l of 32 P-labeled human α -globin mRNA or pre-mRNA, 4 μ l of unlabeled ribozyme RNA, 2 μ l of 500 mM tris-HCl buffer, pH 7.6, and 10 μ l of H₂O. After heating at 96 C for 2 min and quenching in ice water for 3 min, 1 μ l of 200 mM MgCl₂, 0.5 μ l of 200 mM dithiothreitol (DTT), and 1.5 μ l of 39 u/ μ l RNasin (Promega) were added. The ratio of substrate mRNA or pre-mRNA to ribozyme was about 1:4. The reaction mixtures were incubated at 37 C for 15 min, then mixed with 40 μ l of gel loading buffer II [80% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol and 2 mM ethylenediaminetetraacetate (EDTA)]. For analysis of the cleavage results, 10–20 μ l of the samples were loaded on 8 M urea-5% polyacrylamide gels, and electrophoresed at 250 V for 2–2.5 h. The gels were subjected to autoradiography using Kodak XAR-5 film.

Plasmid pBluescript II KS(+) was separately digested with Xma I, Xho I, BssH II, Afl III and ApaL I. The products were used as templates and the 32 P-labeled RNA size markers with length 57, 97, 149, 510, and 824 nt were synthesized using the T7 RNA polymerase *in vitro* transcription system mentioned above.

Cell Culture, Transfection, and Separation of Single Cell Colonies

The human erythroleukemia K562 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (prime), N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), glutamine, penicillin, and

streptomycin, and were maintained at a cell density between 5×10^4 cells/ml and 10^6 cells/ml.

Either the liposome method or electroporation was used to transfect K562 cells. For lipofection, 3 and 7 μ l of Reagent DMRIE-C (1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide, Life Technologies, Gaithersburg, MD) were mixed with 0.5 ml of reduced serum medium OPTI-MEM I (Life Technologies) and 10 μ g of plasmid DNA was mixed with 1.25 ml of OPTI-MEM I. Then, 0.5 ml of DNA-OPTI-MEM I (containing 4 μ g plasmid DNA) was added to each DMRIE-C-OPTI-MEM I, mixed gently and placed at room temperature for 30 min. To each lipid-DNA mixture, 0.2 ml of washed K562 cells (2×10^6 cells) was added and incubated at 37 C and 5% CO₂ for 5 h, then 2 ml of fresh RPMI 1640 medium containing 20% fetal bovine serum was added and the cells were incubated at 37 C and 5% CO₂ for another 24 hours. After that, the cells were spun down and resuspended in 10 ml of fresh RPMI 1640 medium containing 400-760 μ g/ml of G418 (Geneticin, Life Technologies) and incubated at 37 C and 5% CO₂ for 6-12 days. For transfection by electroporation, 5×10^7 K562 cells were electroporated with Gene Pulser (Bio-Rad, Melville, NY) at 250 volts and 960 μ F, with amounts of DNA plasmid ranging from 10 to 30 μ g. For stable transfections, G418 (Cellgro, Herndon, VA) was added at 700 μ g/ml 48 hours after electroporation and the cells were cultured an additional 10-14 days. Cells were then transferred to Petri dishes where single colonies were allowed to grow and then isolated.

The transfected cultures were either used directly in an RT-PCR assay or else in single cell colony selection according to the following procedure. The cultures were carefully diluted with fresh RPMI 1640 medium containing 20% fetal bovine serum to 50-100 cells/ml and cultured in Petri dishes at 37 C and 5% CO₂ for 5-7 days. Single cell clusters of medium size (containing 6-10 cells) were picked up under the microscope and placed separately into wells of a 96-well plate and incubated at 37 C and 5% CO₂ for several

weeks. Each of these single cell colonies in the 96-well plates was allowed to grow up to cover about 1/4 of the well bottom, then transferred into a 25-ml culture flask with 6 ml of fresh RPMI 1640 medium containing 10% fetal bovine serum and 400 μ g/ml of G418. After incubation at 37 C and 5% CO₂ for about 10 days, another 8 ml of medium was added and incubation continued for another 4-6 days. These cultures could be maintained by regular splitting.

³²P-Labeled Antisense RNA Probes for the RNase Protection Assay

A fragment of human α -globin cDNA (containing exons I and II) was placed in the antisense orientation behind the T3 promoter in Bluescript II to form plasmid pKS- α c'- Δ 3'. Using Eag I linearized pKS- α c'- Δ 3' as template, a ³²P-labeled antisense α -globin mRNA probe was synthesized using the MAXIscripT T3 kit (Ambion, Austin, TX). Briefly, the reaction mixture (total volume was 20 μ l) contained 1 μ g of linearized plasmid DNA template, 0.5 mM of ATP, GTP, and CTP, 6 μ l of [α -³²P] UTP (800 Ci/mmol, 25 μ M), 2 μ l of 10 x transcription buffer and 2 μ l of enzyme mixture (10 u/ μ l of T3 RNA polymerase, 5 u/ μ l of RNasin) and was incubated at 37 C for 2 h, then 5 μ l of RNase-free DNase (2 u/ μ l) was added and the mixture was incubated for another 15 min. After that, the reaction mixture was loaded on an 8 M urea-5% polyacrylamide gel. The band corresponding to the full length of the synthesized antisense RNA probe was excised from the gel, soaked with 500 μ l of 0.3 M sodium acetate-1 mM EDTA-0.1% sodium dodecyl sulfate (SDS) at 37 C overnight, then 5 μ g of yeast tRNA and 1.2 ml of ethanol were added, and the mixture was placed in a dry-ice bath for 1 h. The full-length ³²P-antisense RNA probe precipitate was spun down, dried and dissolved in 50 μ l of RNase-free water.

The ³²P-labeled antisense human β -actin mRNA probe was prepared in the same manner mentioned above, except using pTRI- β -actin-

human-125 (Ambion) as template, and using 1 μ l of [α - 32 P] UTP and 0.5-3 μ l of 0.2 mM cold UTP in the synthesis reaction.

RNase Protection Assay

Direct Protect kit (Ambion) was used in the RNase protection assay. Unless specifically mentioned, about 10^7 K562 cells of each single cell colony culture were lysed with 750 μ l of Lysis/Denaturation Solution (Ambion). 60 μ l aliquots of the cell lysate were mixed

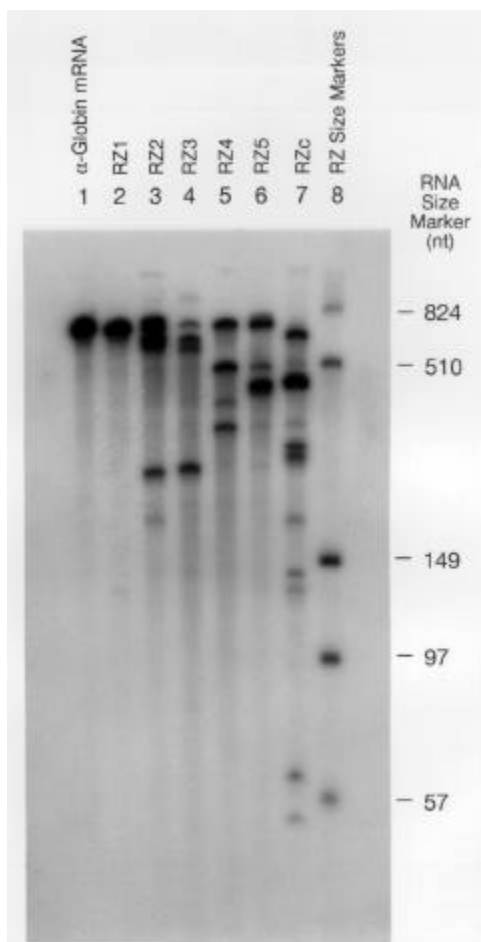


Figure 3. Cleavage of human α -globin mRNA *in vitro*. The 32 P-labeled α -globin mRNA was incubated with different ribozymes at 37 C for 15 min. The experimental conditions are described in Materials and Methods. Lane 1, no ribozyme (i.e., α -globin mRNA only); lane 2, RZ1 (cleavage at 131 nt); lane 3, RZ2 (cleavage at 174 nt); lane 4, RZ3 (cleavage at 227 nt); lane 5, RZ4 (cleavage at 288 nt); lane 6, RZ5 (cleavage at 365 nt) and lane 7, RZc (cleavage at 131, 174, 227, 288 and 365 nt). Lane 8, RNA size markers, from top to bottom: 824, 510, 149, 97 and 57 nt.

with 3-6 μ l of 32 P-labeled antisense RNA probes and incubated at 37 C overnight. The hybridization was performed in probe-excess conditions as the probe RNA added to each sample was about 0.04 pmole estimated by the isotope incorporation in probe synthesis and the radioactivity recovery from the polyacrylamide gel. The hybridization mixtures were then digested with RNase A-T1 and treated with proteinase K according to the Ambion manual. The RNase-resistant double stranded RNA fragments were separated and identified by electrophoresis on a 5% polyacrylamide gel. The radioactivity of the α -globin mRNA fragment or β -actin mRNA fragment was determined by direct counting of the corresponding bands cut from the gel using a Beckman LS 7000 Liquid Scintillation Instrument. The relative amount of α -globin mRNA in a single colony cell culture was calculated using β -actin mRNA as an internal standard or normalizing to the amount of cultured cells.

Quantitative RT-PCR Assay

Real-time (RT), fluorescence-based PCR quantitation of α -globin and multi-ribozyme transcripts was performed using the ABI 7700 Sequence Detector (PE Applied Biosystems). Ribosomal RNA S16 was also quantitated and used to normalize the α -globin and ribozyme transcripts. The sequences used for the primers and probes were: α -globin forward primer (5'-CTCTTCTGGTCCCCACAGACT-3'); α -globin reverse primer (5'-GCAGTGGCTTAGGAGCTTGAA-3'); α -globin probe (5'-FAM-ACCATGGTGCTGTCTCCTGCCG-TAMRA-3'); ribozyme forward primer (5'-AAAACAGGAACATCCTGCATGC-3'); ribozyme reverse primer (5'-CAGGCCACCCTCAACCG-3'); S16 forward primer (5'-ACATCCGTGTCCGTGTAAAGG-3'); S16 reverse primer (5'-TTTGGA-GATGGACTGACGGATA-3'); and S16 probe (5'-TET-AAATCTGGGCCACGTGACCACCA-TAMRA-3'). Total RNA was extracted from 10^7

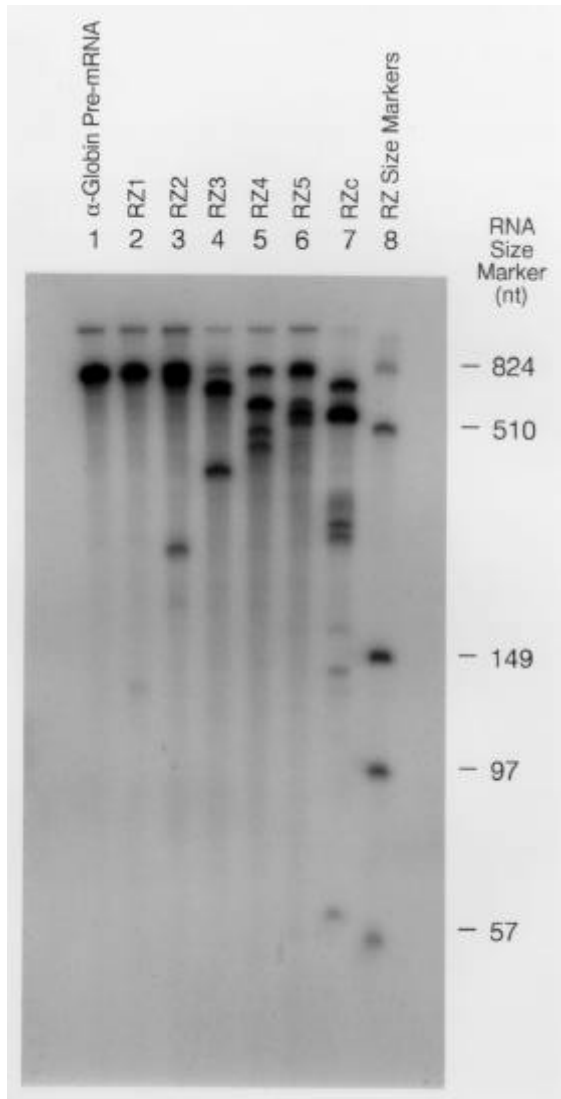


Figure 4. Cleavage of human α -globin pre-mRNA *in vitro*. The 32 P-labeled α -globin pre-mRNA was incubated with different ribozymes at 37 C for 15 min. The experimental conditions are described in Materials and Methods. Lane 1, no ribozyme (i.e., α -globin pre-mRNA only); lane 2, RZ1; lane 3, RZ2; lane 4, RZ3; lane 5, RZ4; lane 6, RZ5, and lane 7, RZc. Lane 8, RNA size markers, from top to bottom: 824, 510, 149, 97, and 57 nt).

cultured cells (Quickprep, Amersham Pharmacia, Piscataway, NJ) and 50 ng of the total RNA was used for cDNA synthesis (Superscript II, Life Technologies). With 2 μ l of cDNA as templates, the PCR was performed by 15 sec at 95 C, 1 min at 60 C, for 40 cycles. The quantitation of α -globin and S16 and relative quantities of α -globin were calculated based on determination of the amplification cycle at which fluorescence intensity

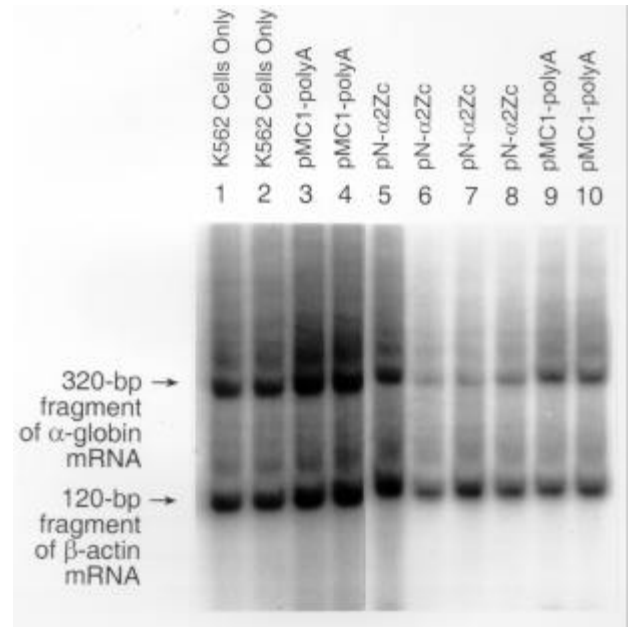


Figure 5. Levels of α -globin mRNA in human erythroleukemia K562 cells transfected with the multi-ribozyme gene directed by the human α -2-globin promoter. The gel shows the result of the RNase protection assay in which the upper band was the α -globin 320-bp fragment and the lower band was the β -actin 125-bp fragment. The experimental conditions are described in Materials and Methods. K562 cells, lanes 1 and 2; K562 cells transfected with the pMC1polyA vector only, lanes 3, 4, 9, and 10; K562 cells transfected with pN- α 2Zc, the multi-ribozyme directed by the human α -2-globin promoter, lanes 5, 6, 7, and 8.

exceeds threshold (threshold cycle or CT) based on the background fluorescence as described in User Bulletin #2 (PE Applied Biosystems). Fluorescence is produced upon cleavage of the

Table 1. The Amount of α -Globin mRNA in Human Erythroleukemia K562 Cells Transfected with Multi-ribozyme Determined by the RNase Protection Assay

	K562 cells transfected with		
	No DNA	pMC1polyA (control)	pN- α 2Zc (ribozyme)
α -globin mRNA ¹	0.983	0.947	0.461
	± 0.006	± 0.040	± 0.196
Relative amount of α -globin mRNA	100%	95.6%	46.6%
α -globin mRNA reduced by		4.4%	53.4%

¹The α -globin mRNA level in each K562 cell sample is shown as the ratio of the radioactivity of α -globin mRNA to that of β -actin mRNA. The α -globin mRNA to β -actin mRNA ratio varies with experiment due to labeling efficiency, but this does not affect the determination of the relative amount of α -globin mRNA which is normalized to the control with no DNA transfected.

probe by Taq polymerase when hybridized to the expected PCR product during PCR amplification. The Sybr Green method, which is used to visualize the PCR product directly without added fluorescence probe, was used in the quantitation of multi-ribozyme. Sybr Green (Molecular Probes, Eugene, OR) was added at 1: 30,000 in the PCR master mixture, and the expression of multi-ribozyme was normalized against S16.

RESULTS

Cleavage of α -Globin mRNA by Ribozymes in Vitro

The cleavage activity of ribozymes RZ1-5 against human α -globin mRNA was tested *in vitro*. Human α -globin mRNA was synthesized *in vitro* with T7 RNA polymerase and labeled with ^{32}P using the α -globin cDNA-containing plasmid pKSh α c' described above. Unlabeled ribozymes were similarly synthesized. Ribozyme activity was assayed by mixing the α -globin mRNA with specific ribozymes and analyzing the reaction products by polyacrylamide gel electrophoresis. Cleavage of α -globin mRNA was observed in all ribozyme reactions (Figure 3), but the best cleavage result was found in the reaction with multi-ribozyme RZc, which combines all five ribozymes (Figure 3, lane 7). The effectiveness of multi-ribozyme constructs has also been seen by other investigators (21,22).

The ribozyme's ability to cleave human pre- α -mRNA was determined, though it is uncertain that the original transcript of human α -globin mRNA could come in contact with ribozymes before its splicing inside the nucleus. ^{32}P -Labeled pre- α -globin mRNA transcript was synthesized *in vitro* with plasmid pKSh α 2g Δ 5' and T7 RNA polymerase as described. As with the mature α -globin mRNA, the pre- α -globin mRNA transcript was incubated with specific ribozymes and the reaction products analyzed by polyacrylamide gel electrophoresis. The result, shown in Figure 4, is similar to that for α -globin mRNA. These data

demonstrate that the multi-ribozyme, RZc, was the most effective ribozyme construct.

Reduction of α -Globin mRNA Level in K562 Cells Transfected by Multi-ribozymes

To assess ribozyme activity in intact cells, K562 cells were transfected with plasmid pN- α 2Zc in which the α -2-globin promoter was used to drive the expression of the penta-ribozyme gene RZc. G418 was used to obtain stable transformants of K562 cells expressing the ribozyme gene. The ribozyme activity was assayed by RNase protection of the isolated RNA by a ^{32}P -labeled specific probe to determine the level of α -globin transcripts. The resultant 320-bp protected RNA fragment has been compared with the RNase protection of a β -actin antisense fragment (125-bp) used as control (Figure 5). An approximately 50% decrease of α -globin pre-mRNA has been found in the randomly selected single K562 cell colony cultures transfected with plasmid pN- α 2Zc (Table 1).

The intracellular α -globin mRNA level in single colony cell cultures is widely varied. This may be due to differences in the proportion of cells with active globin synthesis as well as variation in the extent of globin synthesis per cell. Single colony cell cultures were isolated from transfected K562 cells and the level of α -globin mRNA was determined by RNase protection assay. Transfection with pN- α 2Zc reduced the distribution of α -globin mRNA produced in single colony cell cultures. An observation in single colony cell cultures with pN- α 2Zc with different proportions of cells active in globin synthesis as determined by Benzidine staining showed that the α -globin mRNA level was effectively reduced by multi-ribozyme in all these cell cultures (Table 2). The extent of the reduction of α -globin level among the individual single colony cultures with similar proportions of cells active in globin synthesis could still vary (results not shown). This may be caused by differences in transfected ribozyme gene copy number, the site of

Table 2. Effect of Ribozyme in the Amount of α -Globin mRNA in Single Colony Cell Cultures of Human Erythroleukemia K562 Cells: Comparing Single Colony Cell Cultures with a Similar Proportion of Cells Active in Globin Synthesis

Single K562 cell group	A	B	C	D
% cells active in globin synthesis ¹	0.7-1.0	1.2-1.5	2-3	3.5-4.5
α -globin mRNA level ² in cells transfected with				
pMC1neo-polyA (control)	1643	2306	2230	4905
pN- α 2Zc (ribozyme)	726	1102	808	1881
α -globin mRNA reduced by ³	55.8%	52.3%	63.9%	61.7%

¹The proportion of cells active in globin synthesis was determined by counting the single colony cell culture samples after Benzidine staining. The cells active in globin synthesis exhibited blue color after staining.

² α -globin mRNA level was determined by ³²P counts of the α -globin mRNA in 1×10^6 cells.

³Comparing α -globin mRNA level in the control and the ribozyme groups. The α -globin mRNA of the control was set at 100%.

integration in the genome, recombination or rearrangement events, promoter strength, or other factors. Up to two-fold variation in α -globin mRNA in single colony cultures not expressing ribozyme was observed giving a mean ratio value

of 0.29 ± 0.06 ($n = 11$) when results were normalized to the β -actin control. In comparison, a markedly reduced value and a decrease in variation for α -globin mRNA were obtained for single colony cell cultures stably transfected with pN-SVZc with a mean ratio value of 0.19 ± 0.02 ($n = 9$). These data suggest that multi-ribozyme was able to reduce the level of α -globin in cells with varying levels of α -globin production.

To increase ribozyme expression, the LCR was incorporated 5' of the α -globin promoter to yield pK-LCR- α 2Zc. This provided the greatest reduction in α -globin production when transfected into K562 cells. Real-time was used to directly quantitate the α -globin mRNA in transfected cell pools. A decrease of more than 75% of cellular α -globin mRNA was observed compared with a more modest reduction of pN- α 2Zc (Figure 6). The decrease of α -globin mRNA in cells transfected with pN- α 2Zc (Figures 6 and 9) were approximately similar to those from the single cell colonies. This result showed that the multi-ribozyme directed by the LCR enhancer - α -2-globin promoter was very effective in reduction of cellular α -globin mRNA level.

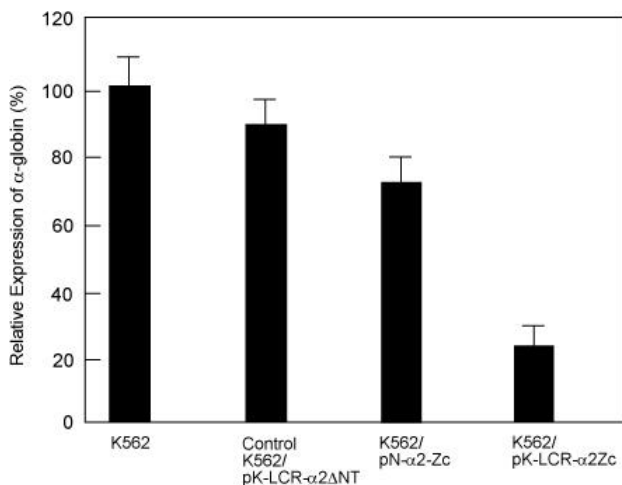


Figure 6. Determination by RT-PCR of levels of α -globin transcripts in K562 cell pools transfected with control plasmid pK-LCR- α 2 Δ NT, and multi-ribozyme constructs pN- α 2Zc (with the α -2-globin promoter) and pK-LCR- α 2Zc (with the α -2-globin promoter combined with the LCR enhancer). The experimental conditions for quantitative RT-PCR are described in Materials and Methods.

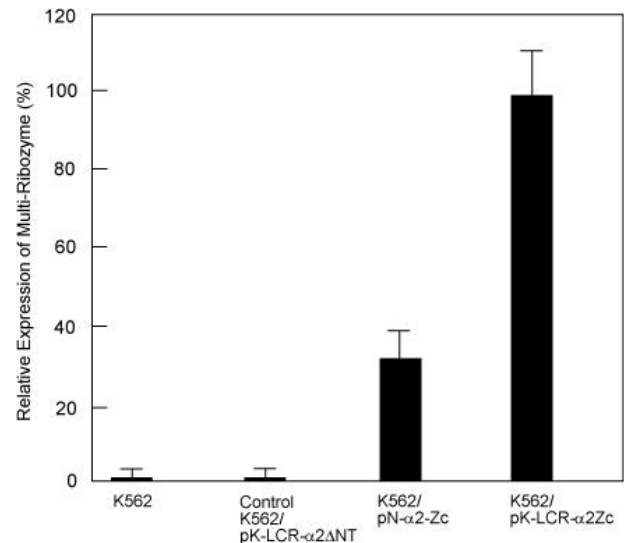


Figure 7. Determination by RT-PCR of levels of multi-ribozyme transcripts in K562 cell pools transfected with control plasmid pK-LCR- α 2 Δ NT, and multi-ribozyme constructs pN- α 2Zc and pK-LCR- α 2Zc.

RT-PCR assays were further used for quantitation of multi-ribozyme transcripts in K562 cell pools stably transfected with plasmids pN- α 2Zc, pK-LCR- α 2Zc, and pK-LCR- α 2 Δ NT (as the control lacking multi-ribozyme). The expression of multi-ribozyme (Figure 7) showed an inverse relationship with the level of the α -globin mRNA expression seen in Figure 6. No multi-ribozyme transcripts were detected in the control groups, and a modest expression of the multi-ribozyme transcripts was found in cells transfected by pN- α 2Zc, while the highest expression of multi-ribozyme mRNA was in the group transfected by pK-LCR- α 2Zc. Combining the results from different assays and plotting the expression of multi-ribozyme against the level of α -globin mRNA present in cells containing either an active multi-ribozyme gene or an inactive control clearly shows that the decrease of α -globin mRNA is correlated with the expression of multi-ribozyme in a dose-dependent manner (Figure 8). This result strongly suggests that the multi-ribozyme transcripts directly contribute to the decrease of cellular α -globin mRNA level.

The multi-ribozyme used in our experiments contains antisense sequence covering exons II and I and intron I of the α -globin gene. In order

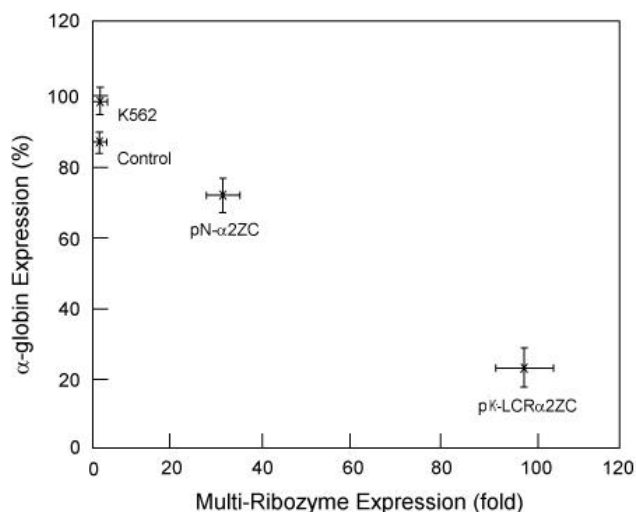


Figure 8. Expression of multi-ribozyme plotted against the level of α -globin mRNA in K562 cells. The RT-PCR results are from different transfection experiments. The experimental conditions for quantitative RT-PCR are described in Materials and Methods.

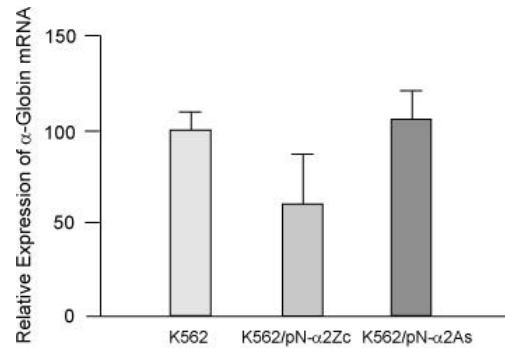


Figure 9. Determination by RT-PCR of levels of α -globin transcripts in K562 cells expressing antisense construct pN- α 2As and multi-ribozyme construct pN- α 2Zc. The experimental conditions for quantitative RT-PCR are described in Materials and Methods.

to determine whether the antisense effect may contribute to a decrease in α -globin mRNA production, the α -globin mRNA in K562 cells stably transfected with both pN- α 2As and pN- α 2Zc was analyzed with RT-PCR. As shown in Figure 9, the level of α -globin transcripts in K562 cells expressing the antisense construct pN- α 2As did not appear to differ from the level in untransfected control cells; in contrast, the level of α -globin transcripts in pooled K562 cells expressing pN- α 2Zc was significantly reduced. These data suggest that reduction in the level of α -globin mRNA is likely mediated by specific ribozyme activity of pN- α 2Zc rather than via an antisense effect.

DISCUSSION

The results presented in this work demonstrate that a multi-ribozyme complex directed against the α -globin transcript can effectively decrease the level of α -globin mRNA in K562 cells. Five single ribozymes against the α -globin mRNA have been designed and *in vitro* measurements suggest that not all of the single ribozymes exhibit the ability to efficiently cleave their target RNAs (results not shown), and that the multi-ribozyme combining all of the five single ribozymes is the most efficient one in the cleaving

the target transcript. When the multi-ribozyme expression plasmid has been transfected in K562 cells, the quantitative results by both RNase protection assays and RT-PCR show that there is a significant decrease of α -globin mRNA in the transfected cells. In the case where the multi-ribozyme is directed by an LCR enhancer – α -2-globin promoter combination, a 78% decrease of α -globin mRNA in the K562 cells was observed. This is a promising result for the possibility of using multi-ribozymes for specific targeting genes in erythroid progenitor cells for treatment of a number of hemoglobinopathies.

A dose-dependent relationship between the decrease of α -globin mRNA and the expression of multi-ribozyme has been indicated by real-time quantitative PCR assays. This strongly suggests that the decrease of α -globin mRNA is caused by the interaction between α -globin mRNA and the multi-ribozyme produced in the K562 cells. Because no antisense effect has been observed, some kind of cleavage of the α -globin mRNA by multi-ribozyme is more likely responsible for the reduction of the α -globin mRNA in the K562 cells.

In this work, the multi-ribozyme expression has been successfully directed by the α -2-globin promoter and furthermore greatly stimulated by the HS4, HS3, HS2, and HS1 sequences of the LCR, a strong erythroid-specific enhancer in the human β -globin gene cluster. This implies that the multi-ribozyme expression can be manipulated in a tissue- and time-specificity, similar to the endogenous α -globin expression and may be synchronous with the α -globin mRNA synthesis in K562 erythroid cells. Further studies in other model systems, such as primary hematopoietic cultures or transgenic mice, will be useful in assessing possible applications of this multi-ribozyme approach for gene therapy in patients with hemoglobinopathies.

Reduction of α -globin transcripts as observed in sickle cell anemia with concomitant α -thalassemia results in reduced intra-erythrocyte concentration of Hb S, and increased red cell survival, hematocrit and whole blood viscosity

(23-25). Although improvement in overall clinical severity has been difficult to demonstrate (25, 26), the increase in red cell survival suggests that combining reduced α -globin production with strategies designed to increase Hb F (to decrease the polymerization potential) could enhance the therapeutic value compared with increasing Hb F levels alone.

The strategy used here to target the α -globin transcript can be readily adapted to target the β -globin transcript in hemoglobinopathies, such as sickle cell anemia or thalassemia. The relatively small size of the multi-ribozyme, 500 nucleotides, is advantageous for its potential use to supplement gene therapy vectors targeted to increase the production of a non- β globin, such as γ -globin. Therapeutic efforts for sickle cell anemia based on induction of normal β -globin or γ -globin expression are intended to reduce the polymerization potential of the sickle erythrocyte. However, for any therapies designed to increase the proportion of normal or fetal hemoglobin, the resultant effect on total intra-erythrocyte Hb and Hb concentration must be considered because of the strong dependence of Hb S polymerization potential on Hb concentration (27). The multi-ribozyme strategy presented here suggests a method not only for targeting specific globin chain production, but also for maintaining balanced α -like and β -like globin gene expression when used in combination with gene targeting designed to increase other β -like or γ -like expression.

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