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Targeted gene conversion in a mammalian CD34$^+$-enriched cell population using a chimeric RNA/DNA oligonucleotide

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Abstract Gene conversion of genetically inherited point mutations is a fundamental methodology for treating a variety of diseases. We tested the feasibility of a new approach using an RNA/DNA chimeric oligonucleotide. The β-globin gene was targeted at the point mutation causing sickle cell anemia. The chimera is designed to convert an A residue to a T after creating a mismatched basepair. In a CD34$^+$-enriched population of normal cells a 5–11% conversion rate was measured using restriction enzyme polymorphism and direct DNA sequence analyses. The closely related δ-globin gene sequence appeared unchanged despite successful conversion at the β-globin locus.

Key words Gene correction · Gene therapy · Stem cells

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Introduction

The human genetic disease sickle cell anemia results from a single base substitution (A→T) in the β-globin gene following a single-gene mendelian mode of inheritance [1–3]. This change, corresponding to the sixth amino acid residue, causes a glutamic acid to valine switch in the β-globin protein. Some individuals with the sickle cell trait have one normal β-globin gene and one sickle cell allele (β^A β^S) and do not exhibit the disease, but their red blood cells become sickled when subjected to very low oxygen pressure in vitro [4, 5]. The pathophysiology of sickle cell anemia is caused by the anomalous properties of sickle cell hemoglobin which has a strong tendency to form intracellular polymers in the deoxygenated state. Those with homozygous alleles (β^S, β^S) do manifest sickle cell anemia. The clinical appearance of this disease includes chronic hemolytic anemia, painful vaso-occlusive crises, and progressive tissue destruction leading eventually to organ failure, although most patients die from a sustained infection. Gene therapy for sickle cell anemia and related thalassemias currently relies on the expression of the β- or γ-globin gene regulated by the locus control region, located over 20 kb upstream from the β-globin gene.

Retroviral vectors or adenoassociated viral vectors [6] are used to introduce some part of this large DNA fragment because the locus control region is required for high-level expression of the transferred gene. Retroviral vectors containing core elements within the hypersensitive sites of the locus control region have been shown to be genetically unstable and often exhibit low transduction efficiencies [7]. Recombinant adenoassociated virus containing the core elements of hypersensitive sites 2, 3, and 4 from the locus control region has been shown to transduce the γ-globin gene with up to 20–40% frequency in CD34$^+$-selected progenitor cells [8]. However, the transferred gene fails faithfully to recapitulate the expression pattern of the normal endogenous gene. The complex and coordinate regulation of globin gene expression in vivo makes it problematic to treat sickle cell

anemia and other thalassemias by gene replacement strategies. The simple correction of specific mutations through the process of homologous recombination would be an advantageous treatment for such hemoglobinopathies. However, the success rate of this strategy in mammalian cells has been hampered by an extremely low frequency of homologous recombination and interference from the illegitimate recombination pathway that takes place independently of DNA sequence homology [9].

Previously we have demonstrated that homologous recombination pathways can be used to induce gene conversion of a point mutation in an extrachromosomal DNA target with a high frequency [10]. The method utilizes a chimeric oligonucleotide composed of a contiguous stretch of RNA and DNA residues in a duplex conformation with double hairpin caps on the ends. The RNA/DNA sequence is designed to align with the sequence of the mutant locus and to contain the desired nucleotide change. An additional feature in the design of the chimeric oligonucleotide is the modification of the RNA residues by 2′O-methylation of the ribose sugar rendering the oligonucleotide resistant to RNaseH activity. Subsequently we demonstrated that the chimeric oligonucleotide, SC1 (see Fig. 1) enabled the conversion of a point mutation present in the β-globin gene of a sickle cell patient to a normal genotype using an Epstein-Barr virus transformed lymphoblastoid cell line [11].

The results generated from these prototype systems enabled a more directed effort on clinically relevant cells and targets. As a first step in this process a population of cells, enriched in $CD34^+$ cells, were chosen since they are likely to be used in ex vivo gene therapy protocols. Because of our interest in hemoglobinopathies induced by point mutations we designed an experimental protocol in which a specific nucleotide in the β-globin gene was targeted for alteration at a site known to cause sickle cell anemia. Due to the relative unavailability of $CD34^+$ cells containing the sickle cell mutation, we utilized $CD34^+$ cells with a normal genotype at the β-globin locus. Hence for feasibility studies we designed a chimeric oligonucleotide that would induce the sickle cell mutation (A→T) and convert the normal gene sequence to a mutated form. Our goal was therefore simply to demonstrate that site-specific gene conversion is possible in a $CD34^+$-enriched cell population.

Materials and methods

Progenitor cell isolation

Normal volunteers were given granulocyte colony-stimulating factor at 300 μg subcutaneously twice a day for 5 five days. On the fourth and fifth days of this therapy they underwent a 4-h stem cell apheresis using a COBE spectra apheresis machine. Mononuclear cells were prepared by density gradient centrifugation on Ficoll-Hypaque (density 1.077 g/ml, Pharmacia, Piscataway, NJ; 2000 rpm, 10 min, room temperature). The majority of the monocytes were removed after adherence (30 min, 37°C in 5% CO_2 in RPMI with 10% FCS), and cells were harvested by swirling to remove cells loosely adherent to the plastic which was washed three times with PBS. This population was incubated with biotinylated murine anti-CD34 antibodies in PBS/1% BSA for 25 min at room temperature at a concentration of 100×10^6 cells/ml. The antibody-treated cells were passed over an avidin column (CellPro, Bothell, WA), and those passing through the column were processed for analysis. Subsequently the column was washed with PBS, and $CD34^+$ cells adhering to the column were recovered by applying a moderate amount of pressure to the column. Final purities were assessed by FACS and were found to be approximately 10%.

Synthesis and purification of oligonucleotides

The chimeric oligonucleotides were synthesized on a 0.2 μmol scale using the 1000-Å wide pore CPG column on the ABI (Applied Biosystems, Foster City, CA) 394 DNA/RNA synthesizer. In this construct the exocyclic amine groups of DNA phosphoramidites (ABI) are protected with benzoyl for adenine and cytidine and isobutyryl for guanine. The 2′-O-methyl RNA phosphoramidites (Glen Research, Sterling, VA) are protected with phenoxyacetyl group for adenine, dimethylformamidine for guanine, and isobutyryl for cytidine. After synthesis the base-protecting groups were removed by heating in ethanol: concentrated ammonium hydroxide (1:3) for 20 h at 55°C. The crude oligonucleotides were purified by polyacrylamide gel electrophoresis, and the sample was mixed with 7 M urea and 10% glycerol, heated to 70°C, and loaded on a 10% polyacrylamide gel containing 7 M urea. After gel electrophoresis DNA bands were visualized by UV shadowing, dissected from the gel, crushed, and eluted overnight in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.5) with shaking. The eluent containing gel pieces was spun through 0.45 μm spin filter (Millipore, Bedford, MA) and precipitated with ethanol. Samples were further desalted by G-25 spin column (Boeringer Mannheim, Indianapolis, IN) and greater than 95% of the purified oligonucleotides were found to be full length as judged by radiolabeling and gel electrophoresis.

Transfection

Cells were re-suspended in RPMI with 10% FCS and 1×10^5 cells/ml were plated in a 24-well plate with each well receiving 1×10^5 cells. The indicated amounts of chimeric oligonucleotide were mixed with 3 μg DOTAP in 20 μl 20 mM HEPES, pH 7.3. The mixture was incubated at room temperature for 15 min then added to the cells. After 16 h at 37°C 5% CO_2, the cells were harvested, pelleted, washed with PBS, and lysed [12].

PCR amplification and analyses

Genomic DNA was amplified for PCR by using primers BGO2 (TCCTAAGCCAGTGCCAGAAGAG) and BGO5 (CTATTGG TCTCCTTAAACCT) and Expand Taq polymerase (Boehringer Mannheim) in a 50 μl reaction at 94°C for 30 s, 52.5°C for 30 s, 72°C for 30 s for 35 cycles to generate a 345-bp fragment. For the δ locus the 5′ primer was BGO6 CTCACAAACTAATGAAA CCCTGC, and the 3′ primer was BGO7 GAAAACAGCCCAA GGGACAG. The program was run at 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s (35 cycles) to generate a 335-bp fragment.

The PCR product was digested with *Dde*I restriction endonuclease (New England Biolabs, Beverly, MA), loaded onto 1.2% agarose gel (1×TBE) and electrophoresed for 2 h at 5 V/cm. The gel was stained for 20 min in the dark in 200 ml of 1×TBE containing 1:20 000 SYBR Green stain (FMC, Rockland, ME) and quantitated by a fluoroimager (Molecular Dynamics, Sunnyvale, CA). The PCR product was spun through a Qiaquick PCR purification spin column (Qiagen, Chatsworth, CA) in H_2O, dried down by vacuum to 5 μl and the concentration determined spectroscopically by OD at 260 nm. The DNA samples (30 ng) were sequenced directly by an automated ABI model 373A DNA sequencing system using the primers described above. We have observed a success rate of 25–30% of cell lots amenable to this form of targeting.

Immunofluorescent staining

RNA-DNA chimeric molecules (SC2) were labeled at the 3′ end using terminal transferase with a digoxigenin-labeled dUTP tail (Boehringer Mannheim). The cells were then transfected with labeled SC2 (2.35 nM) in a 24-well plate for 16 h, as previously described. Coverslips pretreated with polylysine were then placed in the wells. After 1–2 h allowing cells to adhere to coverslips each well was washed three times with PBS. They were then fixed with 3.7% formaldehyde for 10 min; then the samples were permeabilized with 0.05% Triton X-100 in PBS for 10 min and blocked with 4% normal goat serum in 0.05% Triton X-100. Cells were incubated with anti-DIG fluorescein at 37°C for 30 min, mounted and analyzed on a laser scanning confocal microscope (Bio-Rad, MRC-600, Melville, NY) using a ×63 oil immersion lens. Differential interference contrast images were analyzed on a Zeiss Axiovert 100 microscope.

Results

Vector construction and uptake

The design of a chimeric oligonucleotide targeting vehicle is illustrated in Fig. 1. Ten RNA residues flank either side of a five-residue DNA stretch containing the base change desired for correction of the targeted gene. The chimeric oligonucleotide SC1 was designed to correct the point mutation of β^S to β^A (T→A) and therefore has a T residue at the site corresponding to the mutation. The SC2 chimeric oligonucleotide, which has perfect complementarity to the β^S allele, was designed to have no effect on β^S but contains an A residue at the designated position to convert β^A to β^S at the specific site. SC6 is identical in format to SC2 but contains only DNA residues. Chimeric oligonucleotide SC2 contains the RNA stretch complementary to the coding strand of the target whereas SC5 is directed to the noncoding strand. The sequences immediately surrounding the β^A target site are illustrated as well as the sequence of the δ globin gene. This gene has the same sequence as the β^A locus within the target region except for two mismatches that occur in the RNA arms of the chimera (lower case letters) or the all-DNA oligonucleotide (SC6). The δ gene target is useful for the analyses of nonhomologous or nonspecific targeting events.

Previously we have demonstrated that the chimeric oligonucleotide transfected a variety of mammalian cells with a relatively high efficiency [10, 11, 13]. Since transfection was also successful in a $CD34^+$-enriched population, digoxigen-labeled chimeric oligonucleotide was introduced into the $CD34^+$ cell population in order to visualize its uptake. The major targeting effort in this study focused on the conversion of the normal β-globin gene to the sickle cell genotype; hence the SC2 oligonucleotide, which would mediate such a conversion event was used for the uptake experiment. After 16 h the transfected cells were incubated with anti-DIG fluorescein and visualized with a confocal microscope. The results reveal that a majority of cells in this field and within the sample were transfected (Fig. 2). This picture is representative of the seven fields examined.

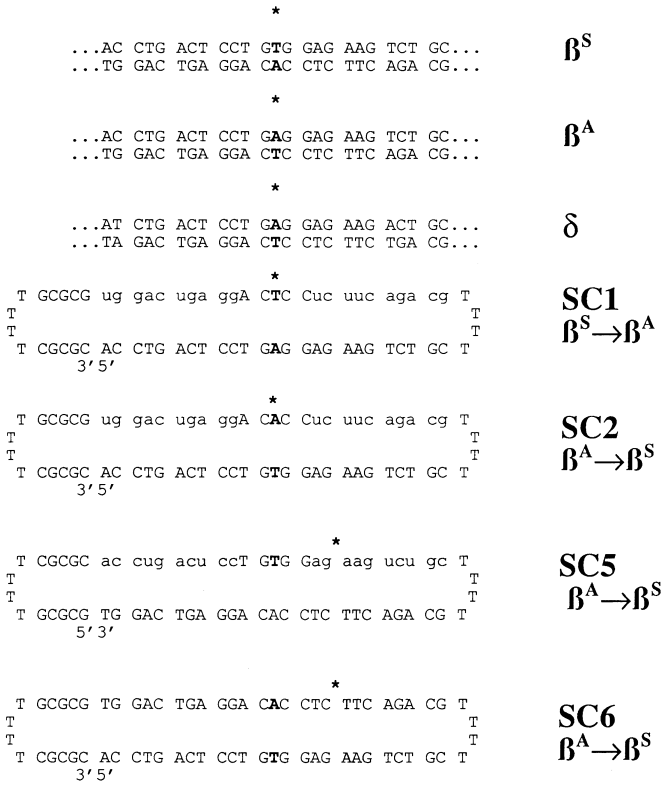

Fig. 1 Sequences of chimeric oligonucleotides and targeted sections of the β^A-, β^S-, and δ-globin genes. *Asterisk*, *boldface*, the site of targeted mutation. The chimeric molecule SC1 aligns perfectly with β^A in the 25-bp region displayed while SC2 matches exactly with the β^S sequence. SC5 targets the noncoding strand of β^S, and SC6 has the same sequence as SC2 except for containing all DNA residues. *Uppercase*, DNA residues; *lowercase*, 2′-O-methyl RNA residues. The β- and δ-globin gene sequence at the targeted site is also presented

Gene conversion

A population of $CD34^+$-enriched cells containing two alleles with β^A genotype was transfected with varying amounts of SC2 in 3 μg/ml of DOTAP. Genomic DNA was isolated 16 h after transfection as described above, and the extent of β^A to β^S conversion was measured by restriction enzyme digestion and by direct DNA sequencing of the PCR fragments. If conversion had occurred in the β^A gene, the restriction enzyme *Dde*I would not be able to cleave the DNA at the positions displayed in Fig. 3a. Genomic DNA isolated from 10^5 cells was subjected to PCR amplification generating a 345-bp fragment by using the two primers BGO2 and BGO5 (see above). The β^A-specific sequence would be cleaved by the restriction enzyme *Dde*I resulting in four fragments of 180, 117, 45, and 3 bp lengths, respectively, while the β^S-sequence would not be cleaved at the sickle locus, thus generating fragments of 297, 45, and 3 bp (Fig. 3a). An increasing level of uncut 297-bp fragment was in fact observed as a function of higher concentrations of SC2, indicating conversion of the β^A to β^S genotype (Fig. 3b). The PCR-generated products were digest-

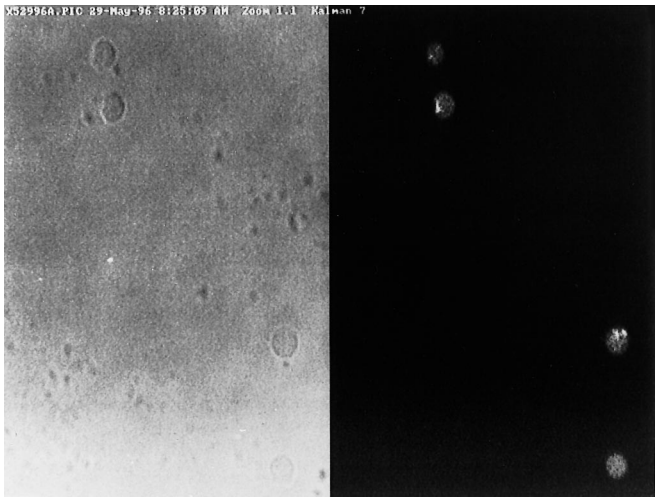

Fig. 2 Cellular uptake of chimeric molecule SC2. Fluorescence images with differential interference contrast images were visualized on samples of a $CD34^+$-enriched population using a digoxigenin-labeled chimeric oligonucleotide. Cells were transfected for 16 h with the modified SC2 and processed for confocal microscopy as described in the text. Images are provided at a magnification of ×63 using oil immersion. *Left*, image of the cell directly; *right*, fluorescent labeling

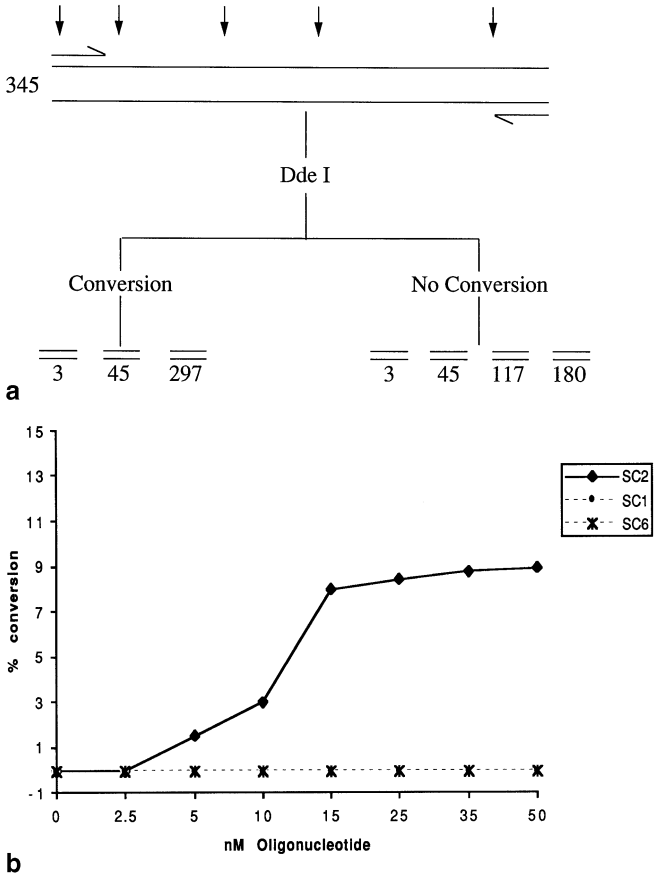

Fig. 3A, B SC2 directs conversion from β^A to β^S in a dose-dependent manner. **A** A 345-bp fragment spanning the site of the point mutation and generated by PCR was digested with the restriction enzyme *Dde*I. Sites of the *Dde*I cleavage are indicated within the genetic sequence. Upon digestion the converted sequence (normal to sickle cell) generates two fragments, while no conversion is scored by the appearance of three fragments. **B** PCR fragments from cells treated with SC1, SC2, or SC6, respectively, were treated with *Dde*I and the products electrophoresed through 1.2% agarose for 2 h. After staining with SYBR Green, the gel was processed for fluoroimaging, and presence of the 297-bp fragment quantitated. The data are plotted as a percentage conversion versus nanomolar concentration of the respective oligonucleotide

ed with *Dde*I electrophoresed through agarose and were stained with SYBR Green. The products on the gel were quantitated by fluoroimaging as reflected by Fig. 3b. A high frequency of conversion was observed at relatively low concentrations of chimeric oligonucleotide in a dose-dependent fashion. No detectable conversion was measured when SC6 (all DNA) was employed, nor was conversion observed in cells treated with SC1, the chimeric molecule which pairs to the β^A site with perfect complementarity. Although the proportional conversion was extensive, we proceeded to analyze the target sequence by DNA sequencing in order to validate the results more directly.

In order to confirm the change (A→T) in normal cells direct DNA sequencing of the 345 bp-fragment was carried out (see above). The $CD34^+$ population containing homozygous β^A alleles was transfected with either 0.47 nM or 23.5 nM SC2 as described. Genomic DNA was isolated and PCR-amplified, and the samples were subjected to automated DNA sequencing. The DNA sequence of β^A alone and β^A treated with the SC1 was shown to be A (as indicated by an arrow), confirming that SC1 did not affect the β^A allele (Fig. 4). In contrast, the DNA sequence of β^A cells, treated with SC2, showed a dose-dependent response in the conversion of A to T. Initially, low levels of the chimera (0.47 nM) display no change while higher levels (23.5 nM) induce a sequence alteration in which a mixture of T and A are seen indicated by the appearance of an N at the designated position (arrow, Fig. 4). Again, no change is observed when the cells are transfected with SC6. Thus we postulate that the chimeric oligonucleotide enabled a sequence specific conversion in CD34-enriched cells.

In mammalian cells homologous targeting events occur at low frequency against a background of nonhomologous events that are more common [14]. Thus a successful gene targeting event requires not only a high frequency but also a stringent specificity to the targeted sequence. The specificity of targeting by chimeric oligonucleotides was tested within the globin gene locus since it consists of a cluster of multiple genes with similar sequences; the sequence of the δ gene contains over 90% similarity to that of the β gene [3]. The central DNA region of the SC2 and the δ gene target resemble the situation involved during SC2 binding to the β-globin locus, but differences exist in two nucleotides among the residues of RNA stretch (Fig. 1). The genomic DNA was isolated from β^A cells treated with increasing doses of SC2, and PCR-amplified utilizing two primers, BGO6 and BGO7 (see above), residing in the δ

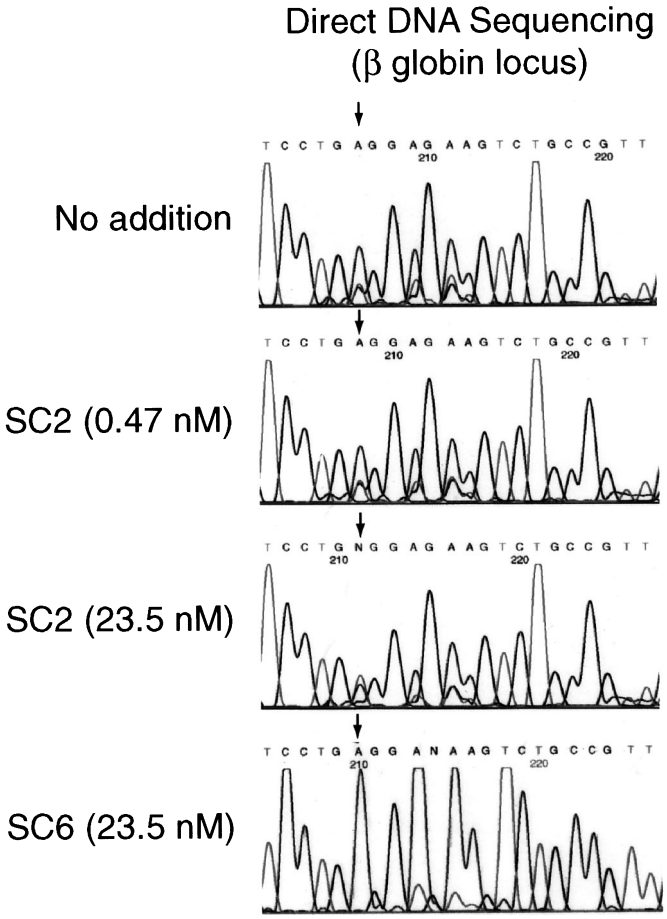

Fig. 4 Direct DNA sequencing of PCR products generated from samples transfected with SC2 or SC6 (β locus). Cells transfected with either SC2 or SC6 were processed 16 h later, and the PCR-based product was sequenced directly. SC2 was added at concentrations of 2.35 and 23.5 nM, respectively, and SC6 was added at 23.5 nM. *Arrow*, the base targeted for change. No addition, no chimeric oligonucleotide added

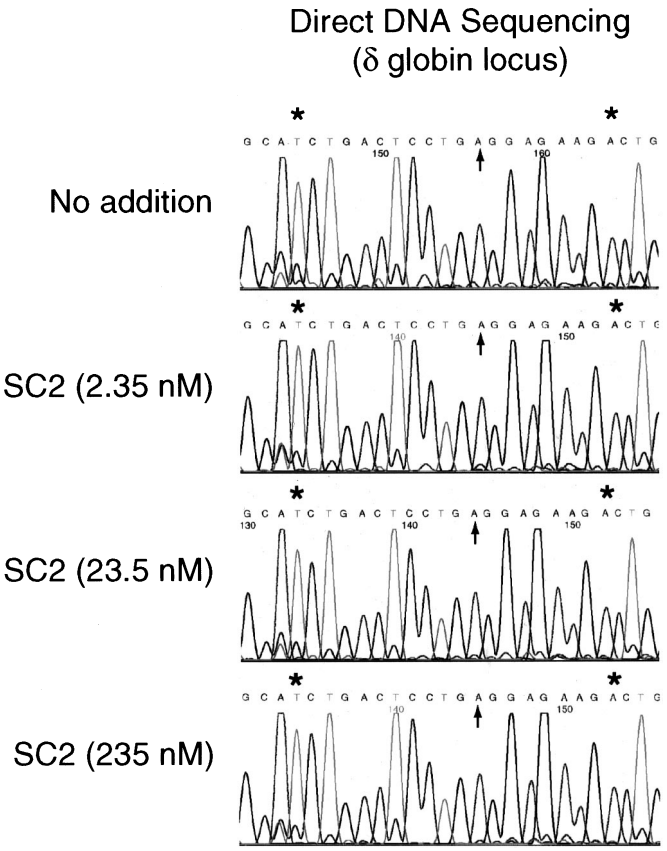

Fig. 5 Direct DNA sequencing of PCR products generated from samples transfected with SC2 (δ locus). Cells transfected with increasing levels of SC2 were processed 16 h later, and the PCR-based product was sequenced directly. SC2 was added at 2.35 nM, 23.5 nM or 235 nM, respectively. *Arrow*, the base designated for change (*A*); *asterisks*, two bases that create a mismatch with the chimeric oligonucleotide. No addition, no chimeric oligonucleotide added

locus. No DNA sequence change in the δ gene was observed even at the highest level of SC2 (Fig. 5). It is not clear whether homologous pairing occurs between the target sequence and chimeric oligonucleotide containing two mismatches in RNA region, but in vitro biochemical analyses suggest that the stability of such joint molecules is substantially reduced when two mismatches are present in the RNA region of the chimera/target complex (A. Cole-Strauss, unpublished data). The lack of gene conversion at the δ locus indicates that the nonhomologous, nonspecific targeting frequency may be low, although extensive experimentation is underway to detect such events.

The strategy behind chimera-based targeting may rely heavily on the interaction between the RNA stretches of the chimera and the complementary region surrounding the target site in the gene. To this point we had based the design of the chimera on complementarity to the coding strand of the gene (SC2). An alternative strategy would encompass designing chimeras that are complementary, vis-à-vis the RNA stretches, to the noncoding strand. Such a chimeric was designed and designated SC5 (see Fig. 1). We repeated the transfection experiments described above with SC2 and SC5 using 1×10^5 cells/ml of a CD34-enriched population. The genomic DNA region surrounding and including the target sites was amplified by PCR using the same strategy described for Fig. 3. The PCR products were treated with *Dde*I, electrophoresed through agarose, stained with SYBR Green and quantitated by fluoroimaging (Fig. 6). In both cases the conversion rate is detectable and exhibits a dose-dependent response. Hence either strand at the target site is amenable to gene conversion events mediated by the chimeric oligonucleotide.

Discussion

These results and those of our previous studies [10, 11, 13] demonstrate that it is possible to generate a point mutation in a gene residing in a chromosome as well as in a plasmid using an oligonucleotide-based strategy. A

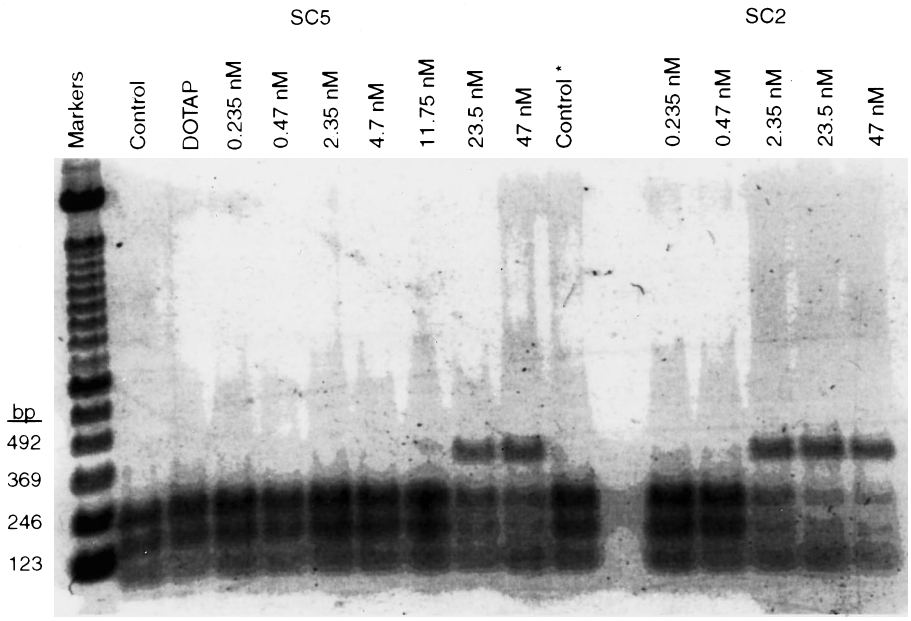

Fig. 6 Quantitation of gene conversion mediated by SC2 or SC5. PCR fragments generated from cells transfected with SC2 or SC5 were treated with *Dde*I, and the digested products electrophoresed through 1.2% agarose for 2 h. After staining with SYBR Green, the gel was imaged by a fluoroimaging system. *Control*, no oligonucleotide or liposome added; *DOTAP*, DOTAP alone added

major modification of the oligonucleotide, incorporation of RNA residues complementary to the region of the targeted strand, appears necessary to make this approach feasible. It is probable that the gene conversion event is mediated by a specific mismatch repair system since SC2 and SC5 were capable of conversion in a specific sequence containing a mismatch with the chimeric oligonucleotide. A chimera (SC1) containing a perfect complementary match with the corresponding target sequence induced no sequence conversion nor did an oligonucleotide containing only DNA (SC6).

The high frequency of correction (10–13%) in chromosomal targeting is a surprising finding because homologous recombination has been reported to be an infrequent event in mammalian cells, ranging from one out of 10^5–10^6 [14–16]. Ingenious procedures have been devised to select or screen for rare successful targeting products, but low absolute frequency of specificity remains a serious limitation in the realm of therapeutics. In contrast, the chimeric oligonucleotide enabled a sequence-specific and high frequency of correction in both chromosomal and extrachromosomal DNA without selection [10, 11]. These data align with recent findings using triplex-mediated gene targeting [17]. The ability to introduce the correction without selection is a particularly attractive feature for gene therapy targeted to stem cells. It is likely that both alleles of the chromosome are accessible for homologous pairing and repair processes since high conversion rates are achieved at elevated concentrations of the chimeric oligonucleotide. Individual cells are now being cloned to measure allelic conversion directly. Moreover, it indicates that RNA/DNA chimeric oligonucleotides may not only enhance the pairing activity [18] but also facilitate mismatch repair to the targeted strand preferentially.

In mammalian cells homologous targeting occurs against a background of nonhomologous events that are more common [9, 15]. Thus we considered the possibility that the chimeric oligonucleotide causes nonhomologous recombination events leading to spurious mutations or random integration. The β-globin gene locus consists of a cluster of multiple genes ε, Gγ, Aγ, δ, and β spanning 50 kb of chromosome 11 (for review see [3]). In particular, the sequence of the δ gene contains over 90% similarity to that of the β-globin gene. Analysis of DNA sequences of the δ locus isolated from the β^A cells treated with SC2 exhibited no alteration within 360 bp surrounding the targeted region (Fig. 4). Thus it appears that two mismatches in the RNA region are sufficient to prevent homologous pairing of the chimeric oligonucleotide and target DNA, indicating a stringent specificity of targeting. We have also examined the possibility that PCR artifacts contribute to the frequency of conversion and under stringent conditions; no evidence for artifactual results was found [19]. Taken together, within our detection limits, we conclude that targeted sequence correction by this oligonucleotide is highly sequence specific and more frequent than random nonhomologous recombination events. It may now be possible to consider the design of therapeutic agents that correct genetic mutations within the context of the chromosome.

One of the real challenges remains to identify the converted target cell in the mixed population. This issue has become more important recently as we have had some difficulty in obtaining cell lots from various mammal donors that are amenable to targeting. For example, out of ten samples only three were found to be amenable to chimeric oligonucleotide-directed targeting. This variability is likely due either to genetic variability of donors or a different level of "targetable" cells in the population. The recent success of Kren et al. [20] in confirming our earlier work for both alkaline phosphatase and β-globin gene targeting in an established cell line (HUH-7) provides a fertile ground for understanding such variability.

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