

Efficiency of Chimeraplast Gene Targeting by Direct Nuclear Injection Using a GFP Recovery Assay

Nam D. Tran,¹ Xiaoming Liu,¹ Ziying Yan,¹ Duane Abbote,^{1,3} Qinshi Jiang,¹
Eric B. Kmiec,² Curt D. Sigmund,³ and John F. Engelhardt^{1,3,*}

¹Department of Anatomy and Cell Biology and the Center for Gene Therapy of Cystic Fibrosis and Other Genetic Diseases, University of Iowa College of Medicine, Iowa City, Iowa 52242-1109

²Department of Biological Sciences, Delaware Biotechnology Institute, University of Delaware, Newark, Delaware 19716

³Department of Internal Medicine, University of Iowa College of Medicine, Iowa City, Iowa 52242-1109

*To whom correspondence and reprint requests should be addressed. Fax: (319) 335-6581. E-mail: john-engelhardt@uiowa.edu.

Traditional RNA-DNA chimeric oligonucleotides (chimeraplasts), composed of a continuous stretch of RNA and DNA residues in a duplex conformation, have been shown to correct single-base mutations in episomal and genomic DNA both *in vitro* and *in vivo*. In the current study, we have compared the efficiency of single-base pair correction between a traditionally designed chimeraplast (covalently linked duplex) and hybrid chimeraplasts (noncovalent duplexes formed from stretches of RNA and DNA nucleotides synthesized individually and hybridized *in vitro*). Six hybrid chimeraplasts of identical length were constructed with various lengths of target homology and strand location of the desired nucleotide change. These constructs were evaluated for their ability to correct a point mutation in the gene encoding recombinant enhanced green fluorescent protein (eGFP) that rendered the protein nonfluorescent. A plasmid encoding this mutant eGFP gene and a chimeraplast were co-introduced directly into the nuclei of primary fibroblasts by microinjection. As shown by the recovery of eGFP fluorescence, three of the six hybrid chimeraplasts demonstrated the ability to mediate gene correction (0.4–2.4%). Covalent joining of RNA and DNA strands in chimeraplasts was not necessary for correction of DNA mutations. However, the strand placement of the desired nucleotide change and the length of nonhomologous sequences flanking target nucleotides played a crucial role in the efficiency of chimeraplast-mediated gene correction. Despite the ability of certain chimeraplast designs to correct point mutations in episomal plasmids, targeted correction of integrated copies of the mutant eGFP transgene was unsuccessful in primary fibroblasts. These results demonstrate that, although chimeraplasts are fairly effective at targeting episomal DNA in primary cells, further optimization is required to increase the efficiency for targeting integrated genes.

Key Words: gene targeting, RNA/DNA oligonucleotides, gene therapy, fibroblasts

INTRODUCTION

Site-specific correction of DNA mutations at their endogenous genomic loci is considered the most direct and effective means of treating genetic diseases by gene therapy. However, achieving this goal has been hampered by the difficulty in developing vectors. One recent promising technology that has emerged for targeted correction of single-base pair mutation employs chimeric RNA-DNA oligonucleotides, or chimeraplasts. These molecules are synthesized as a continuous stretch of RNA and DNA nucleotides homologous to the target DNA sequence, with a 3' G+C-rich clamp and poly(T) hairpin caps at both ends [1,2]. These chimeraplasts have been demon-

strated by many investigators to correct a number of genetic diseases both *in vitro* and *in vivo* [3–7]. Chimeraplasts are designed so that all the DNA and RNA residues line up perfectly with the targeted gene except for a single base within the DNA residues [8,9]. Studies have suggested that this single mismatched base pair in the targeted gene will be recognized and corrected by the cellular DNA repair machinery [1,2,9]. Despite the promise of chimeraplasts as DNA targeting agents, variable results in the efficiency of these molecules have hampered enthusiasm for their many uses in gene therapy. One aspect, thought to be at the foundation of this variability, is the ability of various laboratories to synthesize and purify covalently joined RNA-DNA oligonucleotides to homogeneity with the

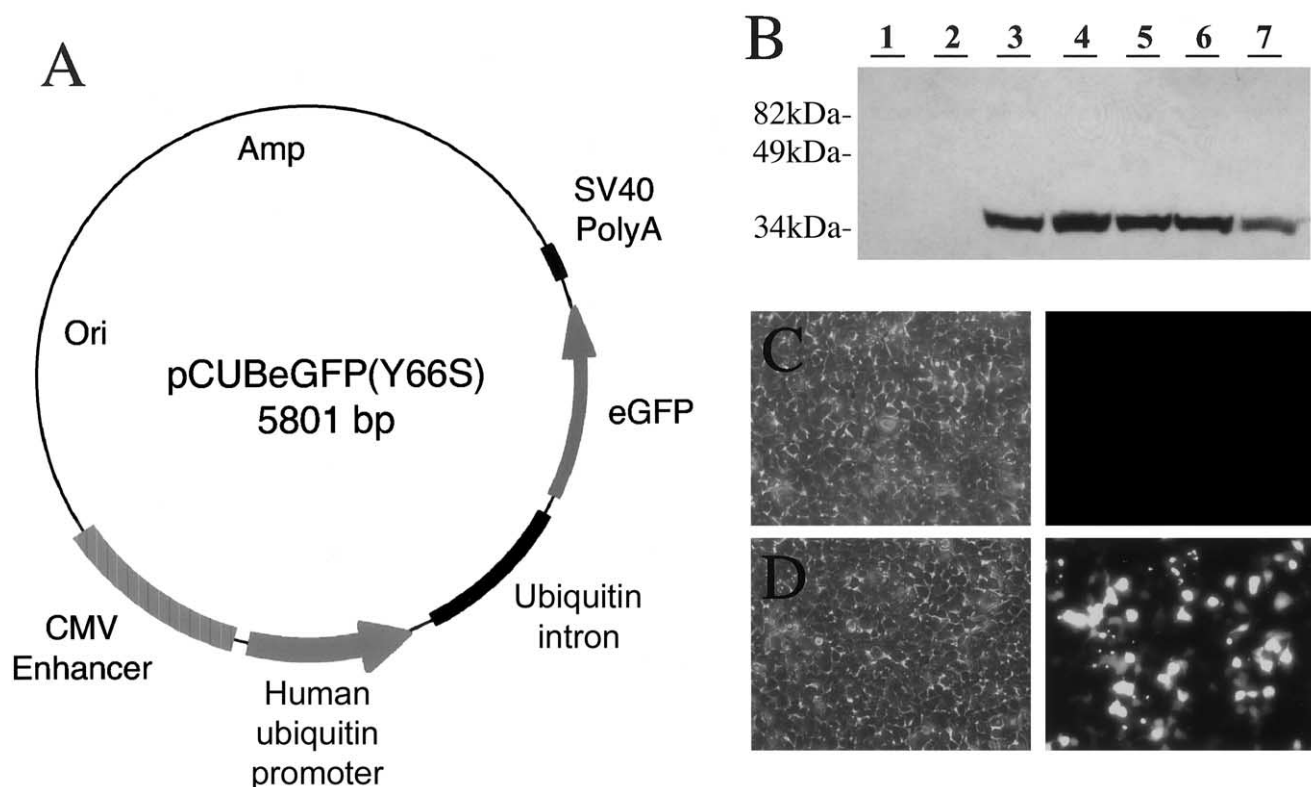


FIG. 1. Map of the mutant pCUBeGFP/Y66S plasmid and expression of mutant eGFP. (A) The mutant eGFP gene was cloned into pUC18 and driven by the CMV IE enhancer and the human ubiquitin promoter as described in the Materials and Methods. The plasmid was sequenced to confirm the A→C mutation (Y66S). (B) Expression of mutant eGFP in 293 cells after transfection. In four samples (lanes 3–6), cells that were transiently transfected with the pCUBeGFP/Y66S plasmid were isolated and subjected to western blot analysis. Negative (lane 2) and positive (lane 7) controls were transfected with plasmids encoding the *lacZ* gene or the wild-type eGFP gene, respectively. Lane 1 represents reagent controls. As demonstrated, a large amount of mutant eGFP was detected in all samples evaluated. 293 cells transiently transfected with either the mutant pCUBeGFP/Y66S (C) or wild-type pCUBeGFP (D) plasmids were evaluated for eGFP fluorescence. As shown, no evidence of fluorescence was observed in cells that were transfected with the pCUBeGFP/Y66S plasmid. In contrast, eGFP fluorescence was readily detected in cells transfected with the wild-type plasmid.

proper secondary structure [1,10]. Additional variations are likely due to alterations in the transfection efficiencies that affect the concentration of targeting agent in the nucleus. The use of molecular PCR endpoints to evaluate gene conversion events has also increased the need for functional readouts of DNA targeting.

The current study has developed a sensitive eGFP functional assay to evaluate chimeraplasts targeting a single-base pair mutation at the cellular level. This assay was used to compare the efficiency of several chimeraplast designs using single-cell nuclear injection and to evaluate fluorescence recovery from a mutant eGFP target plasmid. In contrast to more traditional methods of introducing chimeraplasts into cells (that is, transfection), this direct nuclear injection approach allowed for accurate quantification of targeting efficiencies as a function of the ratio of target to chimeraplast. These studies showed that covalent joining of RNA and DNA strands in chimeraplasts is not required for DNA repair. Furthermore, the studies

demonstrate that regions of non-target homology in the hairpin loop of chimeraplasts are required for gene conversion. However, despite the efficiency of chimeraplasts to target episomal DNA, attempts to target integrated mutant eGFP sequences in primary transgenic mouse fibroblasts failed. Hence, further optimization of the parameters necessary for targeting integrated genes with chimeraplasts is necessary.

RESULTS AND DISCUSSION

In the current study, a recombinant mutant eGFP gene was employed as a marker for chimeraplast-mediated gene correction. Specifically, a single-base point mutation (A→C) was introduced into the wild-type eGFP gene, thus switching the Tyr66 (TAC) to Ser66 (TCC). This specific mutation completely inactivates eGFP fluorescence, as evaluated by the transient transfection of 293 cells with mutant or wild-type pCUBeGFP (Fig. 1). Expression of

eGFP(Y66S) was readily detected by western blot analyses in these experiments (Fig. 1).

To compare the efficiency of a single-base pair correction between the traditional chimeraplast and hybrid chimeraplasts, six different hybrid chimeraplasts were formed by *in vitro* hybridization of different DNA and RNA oligonucleotides: chimeraplasts 1 and 2 had standard 6- to 10-bp G+C-rich hairpin caps flanking 34 bp of target homology, chimeraplasts 3 and 4 extended target homology (50 bp) to the T-loop with no nonhomologous sequence in the hairpin caps, and chimeraplasts 5 and 6 had short 4-bp G+C hairpin caps flanking 42 bp of target homology (Fig. 2). Chimeraplasts 1, 3, and 5 have the desired nucleotide change located only on the DNA strand, whereas chimeraplasts 2, 4, and 6 have the desired nucleotide change located on both the DNA and RNA strands. Gel electrophoresis analysis showed that >90% of the oligonucleotides formed successful complexes after *in vitro* hybridization (data not shown). This is critical because other studies have revealed that impurities in the traditional chimeraplast syntheses could give rise to irreproducible results or negatively affect gene correction efficiency [1,10]. Hybrid chimeraplasts and the eGFP(Y66S) mutant target plasmid were directly introduced into the nucleus of primary fetal fibroblasts by nuclear microinjection. This technology allowed for the strict regulation of the number of chimeraplast and target plasmid molecules introduced into the nucleus using flow rates and injection time controls [11].

Three of the six hybrid chimeraplasts demonstrated the ability to mediate gene correction based on the recovery of eGFP fluorescence (Figs. 3A and 3C). Specifically, at 5 days post-microinjection, $2.4 \pm 0.2\%$, $1.0 \pm 0.1\%$, and $0.4 \pm 0.1\%$ of the fibroblasts that were injected with chimeraplast 1, 2, and 5, respectively, expressed the wild-type protein, whereas no correction was detected in fibroblasts injected with chimeraplasts 3, 4, and 6. A representative photograph of successful gene correction by hybrid chimeraplast 1 is shown in Figure 3C. These results suggest that the length of nonhomologous sequences in the hairpin caps flanking target nucleotides is essential to chimeraplast-mediated gene correction. For example, chimeraplasts 3 and 4, which had the longest length of target homology and no nonhomologous sequence near the hairpin caps, demonstrated no correction. This lack of activity occurred despite similar G+C content in the hairpin caps as seen in the more effective chimeraplasts (1 and 2). These findings support the notion that hairpin caps may contribute to the formation of the double-D loop structure required for effective gene correction [1,2,9] and also suggest that stability of the hairpin loop may not be the primary determinant for effective correction. Interestingly, results also demonstrated that strand placement of the desired nucleotide change also affected activity of chimeraplasts. For example chimeraplast 1, which has the desired nucleotide change located only on the DNA

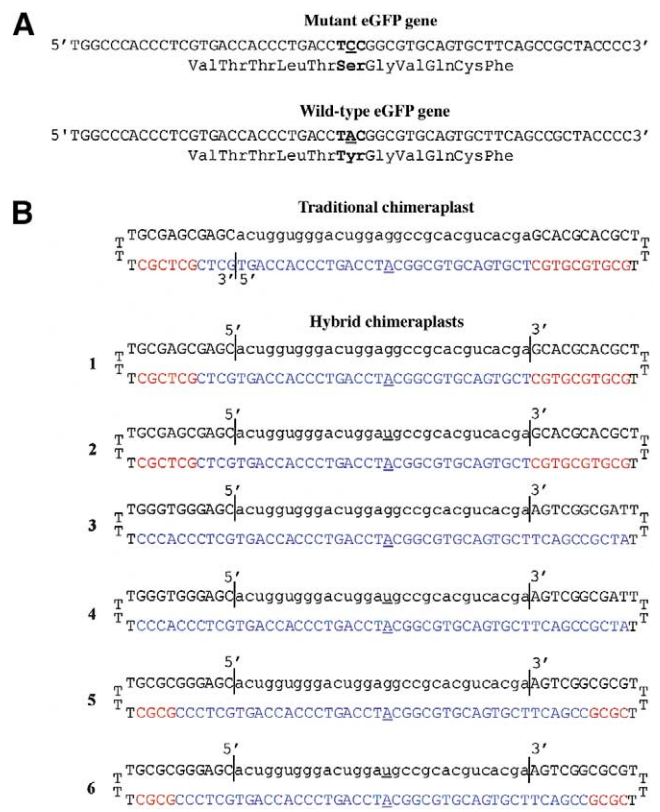


FIG. 2. Chimeraplasts evaluated for correction of the mutant eGFP gene. (A) Target sequences for the mutant and wild-type eGFP transgene. Sense-strand DNA sequence is given with partial corresponding amino acid sequence below. The mutant base alteration (A→C), which gives rise to the tyrosine-to-serine amino acid change is underlined. (B) Chimeraplasts tested for targeting of the eGFP(Y66S) mutation. Junctional breakpoints between RNA (nucleotides in lowercase letters) and DNA (nucleotides in capital letters) oligonucleotides are indicated by a vertical line. The traditional chimeraplast contained a sequence identical to the wild-type eGFP gene between the GC-rich sequences at the hairpin caps and possessed covalently linked RNA and DNA strands. Hybrid chimeraplasts were constructed by *in vitro* hybridization of RNA and DNA oligonucleotides as described in the Materials and Methods. Hybrid chimeraplasts 1 and 2 contained identical GC-rich sequences in the hairpin caps flanking target homology as for the traditional chimeraplast design. In hybrid chimeraplasts 3 and 4, the length of target homology was extended to the T-loop with no nonhomologous sequences in the hairpin caps. Hybrid chimeraplasts 5 and 6 contained an intermediately extended length of target homology into the hairpin caps with a 4-bp CG clamp juxtaposed to the T-loop. Chimeraplasts 1, 3, and 5 have the desired nucleotide change located only on the DNA strand, whereas chimeraplasts 2, 4, and 6 have the desired nucleotide change located on both the DNA and RNA strands. For all chimeraplasts, target homology is marked in blue and hairpin caps are marked in red.

strand, was ~2-fold more efficient at gene correction than chimeraplast 2, which has the desired nucleotide change placed on both the DNA and RNA strands. This was also true for chimeraplasts 5 and 6. This is in agreement with results reported by Gamper and colleagues, which demonstrated the function of the DNA and RNA strands in gene correction and stabilization, respectively [1,8]. Thus, perfect homology of the RNA strand to the target tem-

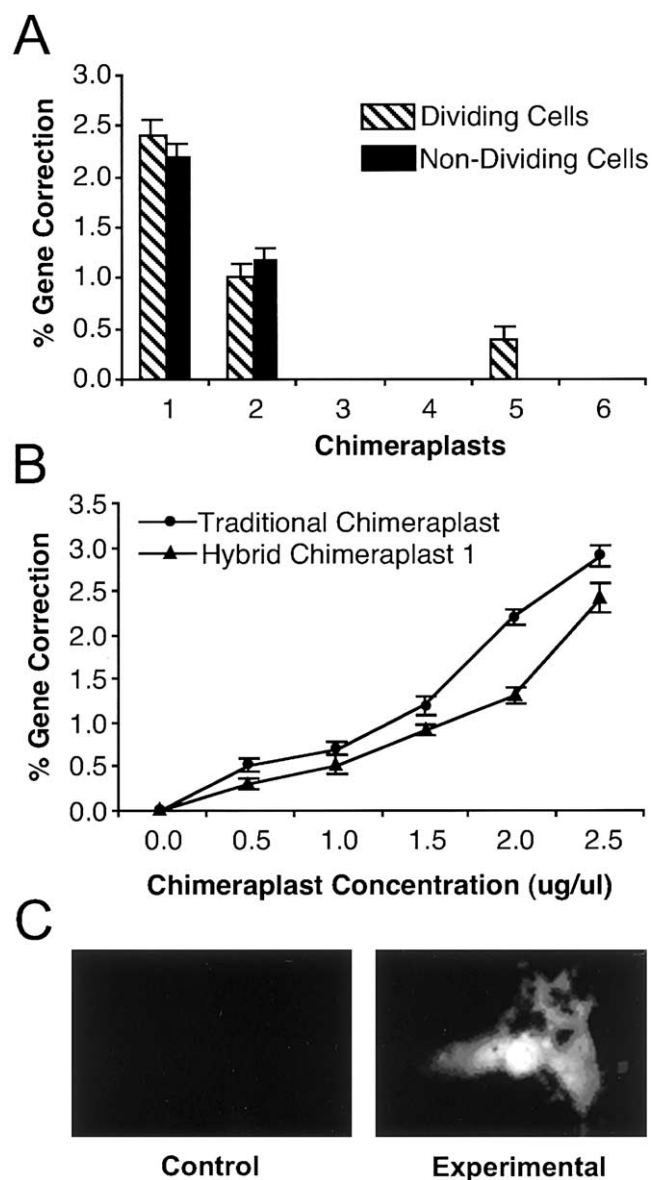


FIG. 3. Efficiency of hybrid chimeraplast-mediated gene correction in fetal ferret fibroblasts. (A) The gene correction efficiency of six different hybrid chimeraplasts was assessed using GFP fluorescence recovery assays. Results represent the mean (\pm SEM) of nine different experiments. In each experiment 400–600 fibroblasts were microinjected with a solution containing both the hybrid chimeraplasts and pCUBeGFP/Y66S plasmids at concentrations of 2.5 $\mu\text{g}/\mu\text{l}$ and 0.5 $\mu\text{g}/\mu\text{l}$, respectively. Microinjected cells were then visualized with fluorescent microscopy for evaluation of evidence for wild-type eGFP expression as described in the Materials and Methods. As shown in (A), hybrid chimeraplasts 1 and 2 were the most efficient at gene correction, suggesting the essential role of the GC ends at the hairpin caps. Furthermore, the similar results between dividing and nondividing cells suggested that the mechanism behind chimeraplast-mediated gene correction is independent of cellular proliferative machinery. (B) Dose-dependent gene correction by the traditional or hybrid chimeraplast 1 after microinjection. Results depict the average \pm SEM of nine different experiments. Fibroblasts were microinjected with pCUBeGFP/Y66S plasmid solution (0.5 $\mu\text{g}/\mu\text{l}$) containing either the traditional chimeraplast

plate is superior and is thought to stabilize the double-D loop structure required for gene correction. In similar experiments, when fibroblasts were introduced with either the DNA or RNA oligonucleotides alone, no evidence of gene correction was detected (data not shown). This is in contrast to previous studies, which demonstrated successful gene correction by short single-stranded DNA oligonucleotides [8,12]. Fundamental differences in oligonucleotide design may contribute to this discrepancy. In contrast to previous reports, the DNA oligonucleotides employed in these studies did not have any phosphorothioate linkages, nor were they capped with 2'-O-methyl uracil at the 3' and 5' ends. This may render the oligonucleotides more sensitive to nuclease activity and limit the duration of available intact oligonucleotide required for gene correction.

When the same experiments were done in growth-arrested fibroblasts, similar results were obtained, thus indicating that gene correction by these hybrid chimeraplasts was equally effective in both dividing and nondividing cells (Fig. 3A). These results suggested that the mechanism of chimeraplast-mediated gene correction through nuclear microinjection is independent of the cellular proliferation machinery. Additionally, the ability to mediate gene correction in quiescent fibroblasts may extend the application of hybrid chimeraplasts to correct mutations in other nondividing cells such as neurons and stem cells. When directly compared, the efficiency of the optimal hybrid chimeraplast 1 was similar to that seen with the traditional structure of a covalently linked chimeraplast (Fig. 3B). As can be seen in this figure, there was a direct correlation between chimeraplast concentration and gene correction efficiency for both traditional and hybrid chimeraplasts, with the highest level of gene correction at 2.5 $\mu\text{g}/\mu\text{l}$. The ratios of chimeraplasts to target plasmids were from 190:1 to 950:1 at chimeraplast concentrations from 0.5 $\mu\text{g}/\mu\text{l}$ to 2.5 $\mu\text{g}/\mu\text{l}$, respectively. Approximately 5000–40,000 molecules of chimeraplast were introduced into the cell nucleus at concentrations between 0.5 and 2.5 $\mu\text{g}/\mu\text{l}$. In all experiments, the concentration of the target mutant plasmid was 0.5 $\mu\text{g}/\mu\text{l}$ (~40 molecules/injection). This target concentration was chosen to ensure that all injected cells expressed mutant eGFP. No evidence of toxicity was observed at any of the chimeraplast concentrations evaluated in these studies. Cell viability upon microinjection ranged between 75% and 85% based on the expression of eGFP from the wild-

or hybrid chimeraplasts at concentrations between 0 and 2.5 $\mu\text{g}/\mu\text{l}$. As demonstrated, the efficiency of both types of chimeraplasts was similar and dose-dependent at all examined concentrations. (C) Representative photomicrograph of wild-type eGFP fluorescence in fibroblast cells after hybrid chimeraplast-mediated gene correction. eGFP fluorescence was readily detected in fibroblasts that were co-injected with pCUBeGFP/Y66S and the hybrid chimeraplast 1 (labeled experimental), and never detected in cells that were injected with pCUBeGFP/Y66S plasmid alone (labeled control).

type plasmid. It is also important to recognize that a chimeraplast concentration of 2.5 $\mu\text{g}/\mu\text{l}$ was the highest that could be achieved with this method because of technical limitations. At a concentration $>2.5 \mu\text{g}/\mu\text{l}$, the injection solution is too viscous to be consistently microinjected. Additionally, the highest efficiencies of correction (2.4%) must be interpreted within the context that ~ 40 target genomes were used to achieve this efficiency. It is presently unclear how many GFP genomes must be corrected to achieve visible fluorescence, and attempts to quantify this by sequencing of rescued genomes were not possible because of the small number of cells injected and low levels of DNA being used. However, if one assumes that a single corrected genome is sufficient to observe fluorescence, the optimal efficiency of correction is actually 40 times lower or 0.06% of target genomes. This is likely an underestimate because studies using five copies of target plasmid per cell failed to demonstrate correction at chimeraplast-to-target ratios of 8000:1. These findings, though not definitive, suggest that more than one genome must be targeted to achieve fluorescence. Hence the targeted efficiency of our optimal chimeraplast likely lies between 0.06% and 2.4%.

Although the targeting of episomal DNA with certain chimeraplasts provided promising results, it was unclear whether similar efficiencies could be achieved with an integrated transgene target. To this end, we generated primary fetal fibroblasts from transgenic embryos harboring ~ 50 copies of the eGFP(Y66S) transgene as determined by Southern blotting (Fig. 4A). These cell lines stably expressed the mutant eGFP protein, as shown by western blotting (Fig. 4B). The ability of our most optimal hybrid chimeraplast (no. 1) to correct eGFP fluorescence following direct nuclear injection was tested in these primary cells. However, no correction was observed in a total of >5000 injected cells. This represents an efficiency of $<0.02\%$ as compared to chimeraplast targeting of 2.4% with episomal plasmids. Given that the chimeraplast-to-target ratios ($\sim 1000:1$) were extremely similar for both episomal and integrated gene targeting experiments, these studies demonstrate that targeting an integrated transgene with chimeraplasts is at least 100-fold less efficient than that for an episomal transgene.

In summary, using a highly controllable reconstitution system of nuclear injection, these studies have demonstrated that covalent joining of RNA and DNA strands in targeting chimeraplasts is not necessary for gene correction. Because synthesis and purification of covalently linked RNA-DNA chimeraplasts is particularly cumbersome and costly, these new data suggest a more cost-effective strategy for generating viable chimeraplasts by *in vitro* hybridization of separate RNA and DNA components. This aspect may be of particular importance in the clinical application of chimeraplasts, in which large quantities must be synthesized. These results also demonstrated that the placement of the desired nucleotide

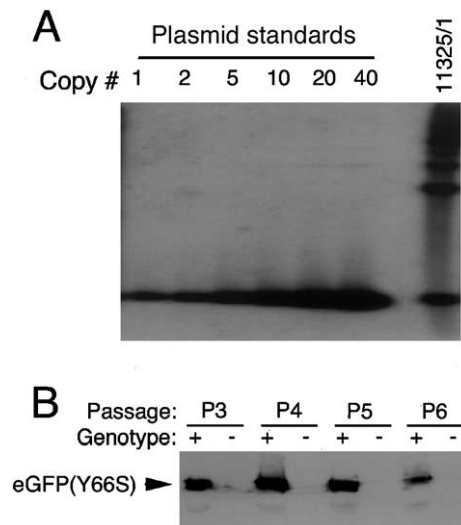


FIG. 4. Characterization of transgenic mouse fetal fibroblasts expressing integrated copies of the CUBeGFP/Y66S transgene. (A) Southern blot analysis of a transgenic CUBeGFP/Y66S mouse line. Copy number standards were produced by reconstitution of pCUBeGFP/Y66S plasmid into mouse genomic DNA and compared to genomic DNA from the CUBeGFP/Y66S line 11325/1. DNA was digested with *Afl*III, and the Southern blot was hybridized to an eGFP DNA probe. (B) Fetal fibroblasts were generated from 13-day transgenic and nontransgenic CUBeGFP/Y66S mice and grown in culture for the indicated number of passages. Western blotting analysis using anti-eGFP antibody was used to confirm expression of the eGFP(Y66S) protein.

change on the RNA or DNA strands and the nucleotide sequences at the hairpin ends play crucial roles in the efficiency of chimeraplast-mediated gene correction. Despite the observable episomal targeting efficiency of chimeraplasts, targeting of integrated DNA sequences appears to be much less efficient. Further research optimizing chimeraplast design may increase the targeting efficiency of integrated sequences. For example, it has recently been reported that the polarity of the targeted DNA strand can substantially affect the efficiency of gene conversion using short single-stranded DNA oligonucleotides (ODN) [12], suggesting that transcriptional influences may play a role in the proofreading repair process. In these studies the highest frequency of repair was observed with ODN in the antisense orientation. Because our current studies have targeted the sense strand, further modification in this regard may further increase gene conversion efficiencies and improve targeted of integrated genes.

MATERIALS AND METHODS

Introduction of the point mutation into the eGFP gene. The eGFP gene from pEGFP-1 (Clontech, Palo Alto, CA) was inserted into pUC18 to form pUC/eGFP. The human ubiquitin promoter from pUB6/V5 (Invitrogen, Carlsbad, CA) and the cytomegalovirus (CMV) enhancer from pIRES (Clontech) were cloned into pUC/eGFP to generate pCUBeGFP. A single-base point mutation (A \rightarrow C) was introduced into the eGFP gene of

pCUBeGFP, thus switching the Tyr66 (TAC) to Ser66 (TCC) using the mutagenesis oligonucleotide 5'-CACCCCTGACCTCCGG CGTGCAGTG-3' and a GeneEditor Mutagenesis Kit (Promega, Madison, WI) according to the manufacturer's instructions. The mutant pCUBeGFP/Y66S was isolated and confirmed by sequencing (Fig. 1).

Detection of mutant eGFP. The 293 cells and fetal fibroblasts were transfected with a control plasmid, mutant pCUBeGFP/Y66S plasmids, or wild-type pCUBeGFP plasmids using Lipofectamine (Invitrogen) and manufacturer's protocols. Fluorescence was directly visualized using a Leica DMR microscope, and cell lysates were evaluated by western blotting with a monoclonal anti-eGFP antibody (Living Colors Peptide Antibody, Clontech) using standard protocols.

Synthesis and hybridization of chimeraplasts. DNA and RNA oligonucleotides were synthesized and purified by PAGE at Integrated DNA Technologies (Coralville, IA) and at Trilink Biotechnologies, Inc. (San Diego, CA), respectively. RNA oligonucleotides contained 2-O-methyl modifications on every second nucleotide. Equal molar concentrations of DNA and RNA components were reconstituted in TE buffer and denatured for 5 minutes at 95 °C. Oligonucleotides were allowed to hybridize at 37 °C for an additional 15 minutes, then hybrid chimeraplasts were evaluated for proper hybridization with nondenaturing gel electrophoresis.

Nuclear microinjection of chimeraplasts and pCUBeGFP/Y66S. Mutant pCUBeGFP/Y66S (0.5 µg/µl) was co-injected with either traditional or hybrid chimeraplasts (0.5–2.5 µg/µl) into primary fetal ferret fibroblasts by nuclear microinjection using an Eppendorf Micromanipulator (Brinkmann Instruments, Ltd., Westbury, NY). Briefly, fibroblasts were isolated from fetal ferrets and expanded in 10% FBS (Hyclone, Logan, UT) DMEM (Invitrogen) with 1% penicillin/streptomycin (Sigma, St. Louis, MO). In some experiments, fibroblasts were treated with mitomycin C (Sigma) to select for nondividing cells according to the manufacturer's instructions. Fibroblasts were plated on CELLocate coverslips (Brinkmann Instruments, Ltd.) according to the manufacturer's instructions and incubated overnight at 37 °C with 5% CO₂. Following incubation, coverslips coated with fibroblasts were plated in a 100-mm dish with 10% DMEM and placed on a heated microscope stage (37 °C). Fibroblasts were then visualized with an inverted microscope (Leica, Bannockburn, IL) and microinjected using Femtotips (Eppendorf). Injected fibroblasts were then allowed to grow in culture for 2–5 days. The gene correction efficiency on viable cells was then evaluated by fluorescent microscopy for evidence of wild-type eGFP expression (Leica).

Generation of transgenic fetal fibroblasts expressing the CUBeGFP/Y66S transgene cassette. Transgenic mice expressing the CUBeGFP/Y66S transgene were generated by pronuclear injection of single-cell mouse embryos by the University of Iowa transgenic core. Transgenic fetal fibroblast lines were generated from 13-day embryos derived from heterozygous matings.

Various embryo-derived fibroblast lines were screened by Southern blotting for copy number, and homozygous fibroblasts were expanded. Western blotting with anti-GFP antibody was used to confirm expression of the eGFP/Y66S transgene. Passage 2 fibroblasts were used for nuclear injection studies as described already.

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