Improved Gene Correction Efficiency with a Tailed Duplex DNA Fragment[†]

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ABSTRACT: A 606-base single-stranded (ss) DNA fragment, prepared by restriction enzyme digestion of ss phagemid DNA, corrects a hygromycin resistance and enhanced green fluorescent protein (Hyg−EGFP) fusion gene more efficiently than a PCR fragment, which is the conventional type of DNA fragment used in gene correction. Here, a tailed duplex, obtained by annealing an oligonucleotide to the ss DNA fragment, was used in the correction. The tailed duplex may be a good substrate for the RAD51 protein, an important enzyme in homologous recombination, which could be the gene correction pathway. The annealing of the oligonucleotides enhanced the correction efficiency of the Hyg−EGFP gene, especially when annealed in the 3′-region of the ss DNA fragment. Both the length and backbone structure of the oligonucleotides affected the gene correction efficiency. This type of gene correction device was also effective for another target gene, the *rpsL* gene. The results obtained in this study indicate that tailed duplex DNA fragments are effective nucleic acids for gene correction.

Gene correction (nucleotide sequence conversion) is an attractive strategy for gene therapy and biotechnology (*1*–*9*). The conversion of the sequence of a disease-causing gene to the normal sequence (gene correction) and the disruption (knockout) of a gene involved in the maintenance of a disease would be achieved with this technology. Single-stranded (ss)[1] oligonucleotides, chimeric 2′-*O*-Me-RNA·DNA oligonucleotides, and triplex-forming oligonucleotides have been examined as chemically synthesized nucleic acids for gene correction. Heat-denatured, 400−800 bp ds PCR fragments, containing the normal sequences, have also been examined for their gene correction abilities by the small fragment homologous replacement method.

Previously, we reported that the gene correction efficiency with an ss DNA fragment (several hundred bases), prepared by restriction enzyme digestion of ss phagemid DNA, was higher than that with a conventional PCR fragment (*10*). Moreover, we found that the ss DNA fragment was integrated into the ds target DNA, suggesting that HR was involved, at least in part, in the gene correction process with the ss DNA fragment (*11*). Therefore, more efficient gene correction could be achieved if we designed novel nucleic acids under the assumption that HR is involved in the process.

A ds ODN containing a 5′-ss tail (5′-tailed ds DNA) is more reactive than an ss ODN in in vitro DNA strand exchange by the human RAD51 protein (*12*). The mammalian RAD51 protein conducts homologous pairing and thus is a very important player in HR. Therefore, an ss DNA fragment with an annealed ODN may be more effective in gene correction than an ss DNA fragment alone. In this study, we annealed oligonucleotides to an ss DNA fragment to make tailed duplexes. The effectiveness of the tailed ds DNA fragments was examined with an episomal Hyg−EGFP fusion gene as a model target. The tailed ds DNA fragments enhanced the gene correction efficiency in position- and length-dependent manners. The sugar structure of the annealed oligonucleotides also affected the efficiency. These results indicate that the gene correction efficiency could be further improved by altering the structure of DNA, when it is designed on the basis of the proposed gene correction mechanism.

EXPERIMENTAL PROCEDURES

Plasmid and Phagemid DNAs. The pTENHEX plasmid and the pBSHES/Sense phagemid are the same DNAs described in our previous study (*10*). The pTENHEX plasmid, containing a mutated Hyg−EGFP fusion gene, was amplified in *Escherichia coli* and was purified with a Qiagen (Hilden, Germany) EndoFree Plasmid Mega kit. The pSSW

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[1] Abbreviations: BNA, 2′-*O*,4′-*C*-methylene-bridged nucleic acid; ds, double-stranded; ENA, 2′-*O*,4′-*C*-ethylene-bridged nucleic acid; HR, homologous recombination; Hyg−EGFP, hygromycin resistance and enhanced green fluorescent protein; LNA, locked nucleic acid; 2′-*O*-Me-RNA, 2′-*O*-methyl-RNA; ODN, oligodeoxyribonucleotide; ss, single-stranded; sem, standard error of the mean.

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Table 1: Oligonucleotides Used in This Study

oligonucleotide	sequence	tailed ds
	DNA	
AS (1−35)	5′-CTTTTTCATGGTGGCGGCGGCTCCGGGGATCTCGA	A35
AS (111−145)	5′-TCGAAGCTGAAAGCACGTGATTCTTCGCCCTCCGA	B35
AS (286−320)	5′-GACACCCTGTGCACGGCGGGAGATGCAATAGGTCA	C35
AS (572−606)	5′-GGTGCCGGACTTCGGGGCAGTCCTCGGCCCAAAGC	D35
AS (587−606)	5′-GGTGCCGGACTTCGGGGCAG	D20
AS (580−606)	5′-GGTGCCGGACTTCGGGGCAGTCCTCGG	D27
5′-XhoI loop	5′-GGCGGCTCCGGGGATCTCGAGCGAAAGC	5′-loop
3′-XhoI loop	5′-GCGAAAGCGGTGCCGGACTTCGGGGCAG	3′-loop
	DNA−BNA/ENA Chimera[a]	
BNA-AS (587−606)	5′-GGTGCCGGACTTCGGGGCAG	BNA20
ENA-AS (587−606)	5′-GGTGCCGGACTTCGGGGCAG	ENA20
	2′-*O*-Me-RNA	
2′-O-Me-AS (587−606)	5′-GGUGCCGGACUUCGGGGCAG	2′-*O*-Me-RNA20
	DNA (*rpsL*)	
rpsL AS (760−794)	5′-CAGATCGAAGTACTCAGTTTAAGCTGGCCTCCATG	

[a] BNA and ENA parts are underlined.

plasmid, containing the normal *E. coli rpsL* gene (*13*), was also prepared in a similar manner.

Oligonucleotides. All ODNs and the ENA oligonucleotide were purchased from Sigma Genosys Japan (Ishikari, Japan) in purified forms. The purified BNA/LNA oligonucleotide was obtained from Thermo Electron (Ulm, Germany). The 2′-*O*-Me-RNA oligonucleotide was synthesized and purified as described previously (*14, 15*). These oligonucleotides were desalted by gel-filtration column chromatography. The oligonucleotides used in this study are listed in Table 1.

Preparation of DNA Fragments for Gene Correction. The *E. coli* JM105 strain harboring the pBSHES/Sense phagemid was cultured overnight, and the phagemid DNA was obtained as described previously (*10*). We prepared the 606-base ss fragment (fSense), for correction of the Hyg−EGFP gene, from the ss pBSHES/Sense phagemid by annealing them with their respective scaffold ODNs, followed by XhoI digestion, as described previously (*10*). The DNA fragment was purified by low-melting point agarose gel electrophoresis and gel-filtration chromatography. Its UV spectrum was measured to confirm its purity and to calculate the yield. The concentration was determined by the molar absorption coefficient of DNA: 1.0 OD_{260} equals 40 μg of ss DNA (*16*). The 795-base ss fragment for "correction" (introducing a mutation) of the *rspL* gene was prepared similarly, and this will be reported elsewhere. The ss DNA fragment was mixed with a 10-fold molar excess of an oligonucleotide. The mixtures were heat-denatured at 98 °C for 5 min and were immediately chilled on ice for at least 5 min. They were then heated at 80 °C for 5 min and cooled slowly to room temperature.

Cell Culture and Transfection. Each DNA fragment (10 pmol) was mixed with 125 ng (25 fmol) of the target plasmid (pTENHEX). An appropriate amount of pALTER-Ex2 (Promega, Madison, WI), which does not affect the gene correction assay, was added to keep the total amount of DNA (the N/P ratio) constant. The total amount of DNA was fixed at 4 μg in this study. Transfection into CHO-K1 cells was carried out with Lipofectamine Plus Reagent (Invitrogen, Carlsbad, CA), according to the supplier's instructions. Forty-eight hours posttransfection, the cells were harvested, and the plasmid DNA was recovered, as described previously (*10*).

Determination of Gene Correction Efficiency. Electrocompetent *E. coli* cells were prepared essentially according to the method described in the literature (*17*). Correction efficiencies for the Hyg−EGFP gene were calculated by dividing the number of EGFP-positive colonies on agar plates containing Hyg by the number of colonies on agar plates without Hyg, as described previously (*10*). Under our conditions, we obtained $\sim 10^4$ total colonies on the plates without Hyg in a single transfection experiment. Correction efficiencies for the *rpsL* gene were calculated by dividing the number of colonies on the plates containing streptomycin by the number of colonies on the plates without the antibiotic, essentially as described previously (*13*).

RESULTS

Effects of the Location of the ODNs. We hypothesized that an ss DNA fragment with an annealed ODN (tailed ds DNA fragment) may be more effective in gene correction than an ss DNA fragment, since tailed duplexes are more reactive than ss ODNs in in vitro DNA strand exchange by the human RAD51 protein, which is involved in HR (*12*). First, we examined the position effects of the ODNs on the correction efficiencies of the Hyg−EGFP fusion gene. A mutated Hyg−EGFP gene in which codon 34 is TGA (termination codon), instead of the normal TCA (Ser) sequence (*10*), was used as the gene correction target. Conversion from the TGA to TCA sequence restores both the hygromycin resistance and EGFP-positive phenotypes. A 606-base ss DNA fragment (fSense) was annealed with 35mer "helper" ODNs complementary to the 5′- or 3′-terminus, or the central part of fSense [fragment A35, D35, or C35, respectively (Figure 1)]. In addition, an ODN complementary to the target region containing codon 34 was used (fragment B35). The annealed DNAs (tailed ds DNA fragments) were cotransfected with the target DNA (pTENHEX) containing the TGA sequence at codon 34 into CHO-K1 cells. Plasmid DNA was recovered from the transfected CHO-K1 cells 48 h after the transfection, and the gene correction efficiency was determined.

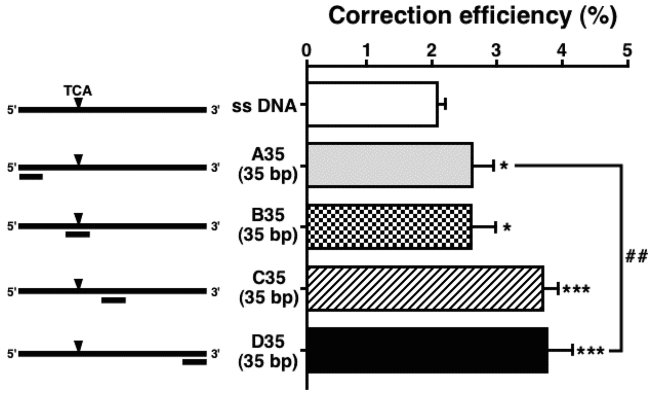

FIGURE 1: Effects of the position of helper 35mer ODNs on the gene correction efficiency. DNA fragments were cotransfected with the target pTENHEX plasmid containing the inactivated Hyg−EGFP gene (400:1 molar ratio) into CHO-K1 cells. The plasmids recovered from the cells 48 h posttransfection were used to transform the *E. coli* BL21(DE3) strain. The gene correction efficiency was determined as described previously (*10*). Bars denote the sem ($n = 5$) (*$P < 0.05$ vs the ss DNA fragment; ***$P < 0.001$ vs the ss DNA fragment; $^{\#\#}P < 0.01$).

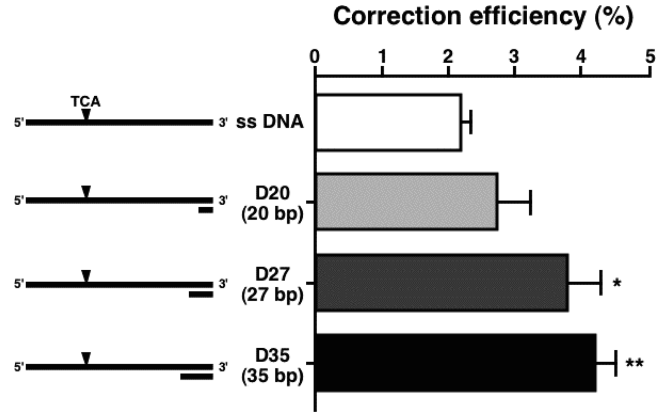

FIGURE 2: Effects of the length of helper ODNs on the gene correction efficiency. The corrections of the inactivated Hyg−EGFP gene were carried out as described in the legend of Figure 1. Bars denote the sem ($n = 4$) (*$P < 0.05$ vs the ss DNA fragment; **$P < 0.01$ vs the ss DNA fragment).

As shown in Figure 1, treatment with a 400-fold molar excess of fSense yielded a gene correction efficiency of ~2%. Annealing with any of the helper ODNs complementary to the various regions of the ss DNA fragment increased the gene correction efficiencies. The improved effects were remarkable with the ODNs complementary to the 3′-terminus and the central region (fragments D35 and C35, respectively). The gene correction efficiencies were ~4%, which is twice the value of the correction efficiency with fSense. Note that the ds region of fragment C35 is on the 3′-side of the target (codon 34). Thus, in agreement with the results from in vitro DNA strand exchange conducted by the human RAD51 protein (*12*), ds DNAs containing a 5′-ss tail (5′-tailed ds DNA fragments) were most effective in gene correction. This may be attributed to the enhanced efficiency of recognition by the RAD51 protein (or other proteins involved in the gene correction) in the transfected CHO-K1 cells. Thus, this result supported our hypothesis. When we annealed a mixture of two helper ODNs, no significant increase in gene correction was observed (data not shown). Helper ODNs complementary to the 3′-terminal region of the ss 606-base DNA fragment were used in the following experiments.

Effects of ODN Length. Next, the effects of the helper ODN lengths were examined. The 5′-tailed ds DNAs containing 20mer, 27mer, and 35mer ODNs complementary to the 3′-terminal region of the ss DNA fragment were assayed. As shown in Figure 2, the gene correction efficiencies depended on the length of the ODNs. The tailed ds DNA fragment containing the 35mer ODN was most effective, followed by the 27mer ODN. On the other hand, complementary helper 40mer and 50mer ODNs did not enhance the gene correction efficiency, as compared with the 35mer ODN (Figure 1 of the Supporting Information). These results indicate that an ~35 bp ds region was long enough to enhance the gene correction efficiency. This length effect may be explained by the duplex stability and/or affinity for RAD51 and/or other HR proteins.

Effects of Modified Oligonucleotides. We next focused on the sugar puckers of the DNA bound to the RAD51 protein. In B-form and A-form DNA, the sugar puckers are of the S type (C2′-*endo*) and N type (C2′-*exo*), respectively (*18*). Nishinaka et al. previously suggested that the sugar pucker in ds DNA bound to the active form of the *E. coli* RecA protein, the bacterial homologue of RAD51, was the N-type conformation (*19*). We noticed that DNA and some modified nucleic acids with the N-type sugar puckers form A-type duplexes. These modified nucleic acids include 2′-*O*-Me-RNA, BNA (also known as LNA), and ENA (*20–23*). Thus, tailed duplexes containing these modified nucleic acid oligomers might be recognized well by the active RAD51 protein. This hypothesis prompted us to examine the effects of 2′-*O*-Me-RNA, BNA/LNA, and ENA as the helper oligonucleotides.

In the case of 2′-*O*-Me-RNA, all of the DNA residues were replaced with 2′-*O*-Me-RNA (Table 1). Due to possible problems in the synthesis and purification, not all of the residues were substituted with BNA/LNA and ENA. Instead, they were introduced near their 3′-end (ss−ds junction), under the assumption that the sugar puckers in the ss−ds junction region were most important for the binding to the RAD51 protein. These oligonucleotides were 20 bases long, because of difficulty in the synthesis of longer ones.

The tailed ds DNA fragment containing the BNA/LNA 20mer exhibited a gene correction efficiency similar to that obtained with the DNA 20mer (fragment D20) (Figure 3). The efficiency was increased, to 4%, when the ENA 20mer was annealed with the ss DNA fragment. In marked contrast, the 2′-*O*-Me-RNA 20mer decreased the gene correction efficiency. Thus, only the ENA 20mer outcompeted the DNA 20mer, in terms of correction efficiency.

The improved gene correction efficiency with the annealed ENA oligonucleotide could not be explained solely by the sugar pucker, because the BNA/LNA and 2′-*O*-Me-RNA oligonucleotides did not exert positive effects, as compared to the DNA 20mer. The fact that the 2′-*O*-Me-RNA 20mer reduced the gene correction efficiency suggests that the duplex stability was important. However, the stabilities of BNA·DNA and ENA·DNA hybrids are comparable (*23, 24*), and this is in contrast to the finding that only the ENA 20mer corrected the target gene more efficiently than the DNA 20mer (Figure 3) (see Discussion). All of the residues in the 2′-*O*-Me-RNA helper oligonucleotide were substituted. This may be a reason for its gene correction efficiency being lower than that of the DNA 20mer.

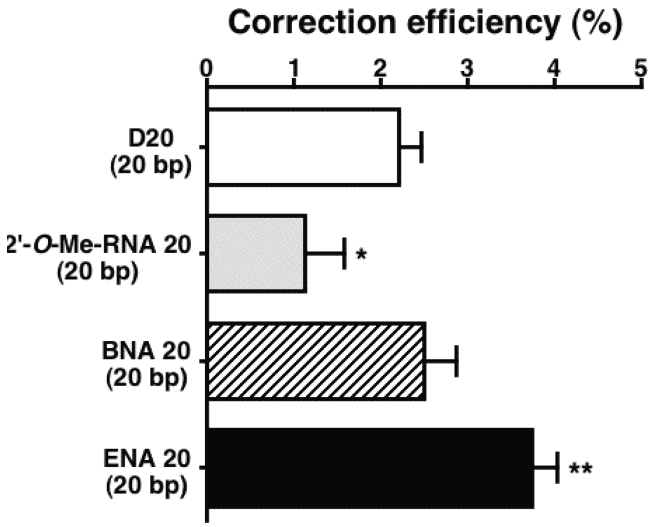

FIGURE 3: Effects of 20mer helper modification on gene correction efficiency. The corrections of the inactivated Hyg−EGFP gene were carried out as described in the legend of Figure 1. Bars denote the sem ($n = 4$) (*$P < 0.05$ vs fragment D20; **$P < 0.01$ vs fragment D20).

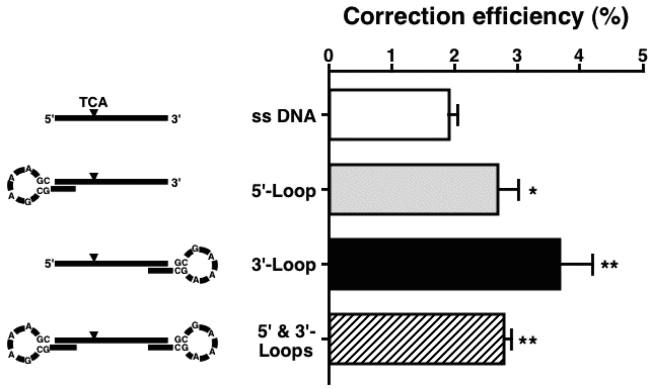

FIGURE 4: Effects of loop-forming ODNs on gene correction efficiency. The corrections of the inactivated Hyg−EGFP gene were carried out as described in the legend of Figure 1. Bars denote the sem ($n = 8$) (*$P < 0.05$ vs the ss DNA fragment; **$P < 0.01$ vs the ss DNA fragment).

Effects of Loop-Forming ODNs. We next focused on the stability of the ss DNA fragments in the cells. The fragment would be degraded by exonucleases. ODNs with a thermally stable loop were annealed with the ss DNA fragment to protect its terminus from exonucleases (Figure 4). These ODNs contained a 20-base sequence complementary to the 5′- or 3′-terminal region of the ss DNA fragment and a stable loop consisting of the GAAA sequence (*25*).

As shown in Figure 4, the annealing of a loop-forming ODN to the ss DNA fragment yielded more efficient gene correction. The enhancement was greater with the 3′-protection. When both of the loop-forming ODNs were annealed with the ss fragment, the gene correction was less efficient than that with only the 3′-protecting ODN.

The annealing of the 3′-protecting ODN yielded a gene correction efficiency of ∼4% (Figure 4). This improvement in the correction might be due to more effective recognition by RAD51 and/or other protein(s) as well as the expected protecting function. In the case of tailed duplexes, the annealing of the "straight" 20mer ODN did not affect the gene correction, and longer ODNs were more effective (Figure 2). Thus, the gene correction might be enhanced when the duplex region is extended from 20 to 35 bp. However, the annealing of loop-forming ODNs containing 20- and 35-base sequences complementary to the 3′-terminal region of the ss DNA fragment resulted in similar gene correction efficiencies (data not shown). Thus, the extension of the complementary region failed to improve the gene correction efficiency, in the case of the loop-forming ODNs.

Microscopic Observation of Gene Correction. As shown above, the annealing of an oligonucleotide to the ss DNA fragment improved the gene correction. The 5′-tailed ds DNAs containing the 35mer ODN, 20mer ENA, and loop-forming ODN, which are complementary to the 3′-terminal region of the ss DNA fragment, corrected the target Hyg−EGFP gene with an efficiency of ∼4% (Figures 1–4). These quantitative data were obtained by counting the EGFP-positive and hygromycin-resistant *E. coli* colonies. Since the target pTENHEX plasmid contains the cytomegalovirus promoter in addition to the phage T7 promoter, the correction of the Hyg−EGFP gene could be observed by counting EGFP-positive CHO-K1 cells by fluorescence microscopy.

We next observed the correction of the Hyg−EGFP gene by the three 5′-tailed ds DNAs with a fluorescence microscope. Transfection of pTENHEX with the 5′-tailed ds DNA containing the 35mer ODN resulted in the appearance of EGFP-positive CHO-K1 cells. The percentage of EGFP-positive cells was 2.8% with this DNA, while it was 1.6% with the ss DNA fragment. Thus, the gene correction by the 5′-tailed ds DNA containing the 35mer ODN was more efficient than that by the ss DNA fragment in the CHO-K1 cells. The yields of EGFP-positive CHO-K1 cells were 3.0 and 2.6% with the 5′-tailed ds DNAs containing the 20mer ENA and the loop-forming ODN, respectively. These percentages would be highly dependent on the introduction efficiency (the percentage of cells that incorporated the target plasmid and the DNA fragment) and the copy number of the corrected plasmid molecules per cell. Although the percentage of EGFP-positive CHO-K1 cells reflects the gene correction efficiency semiquantitatively, the 5′-tailed ds DNAs containing the 35mer ODN, 20mer ENA, and loop-forming ODN corrected the inactivated Hyg−EGFP gene with similar efficiencies.

Correction of Another Target Gene with 5′-Tailed ds DNA. We finally examined whether a 5′-tailed ds DNA containing a 35mer ODN could correct another target more efficiently than ss DNA. The *rpsL* gene encodes the S12 protein, one of the small subunit proteins of the *E. coli* ribosome. Specific mutations of the *rpsL* gene confer a streptomycin-resistant phenotype (*strA*) to bacteria. The expression of the wild-type *rpsL* gene exhibits a dominant phenotype of streptomycin sensitivity over the mutant phenotype. Thus, the *E. coli strA* strain harboring the plasmid with the wild-type *rpsL* gene exhibits the streptomycin-sensitive phenotype, while the *strA* strain harboring the plasmid with the mutated *rpsL* gene shows the streptomycin-resistant phenotype. We chose codon 43 in the *rpsL* gene as the target. The sequence change at this position [AAA (Lys) → ACA (Thr)] can be detected by a phenotypical change, from streptomycin sensitivity to resistance (*13*). The ss sense fragment (795-base) annealed with the 35mer ODN was transfected into CHO-K1 cells together with the target plasmid, and the gene correction efficiency was determined by counting streptomycin-resistant *E. coli* colonies, obtained by transfection with plasmid DNA

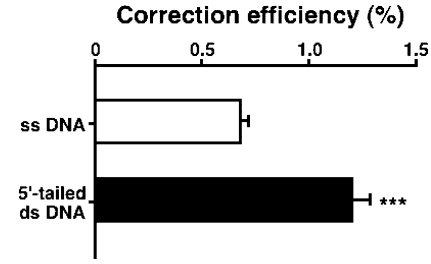

FIGURE 5: Correction efficiencies of the *rpsL* gene with ss DNA and 5′-tailed ds DNA fragments. These DNA fragments were cotransfected with the target pSSW plasmid containing the *rpsL* gene (400:1 molar ratio) into CHO-K1 cells. The plasmids recovered from the cells 48 h posttransfection were used to transform the DH10B *E. coli* strain. The gene correction efficiency was determined as described previously (*13*). Bars denote the sem ($n = 4$) (***$P < 0.001$ vs the ss DNA fragment).

recovered from the CHO-K1 cells. As shown in Figure 5, the *rpsL* gene was corrected by the 5′-tailed ds DNA 1.8-fold more efficiently than by the ss DNA. Thus, this novel gene correction device would be applicable to other genes as well as the Hyg−EGFP gene.

DISCUSSION

In this study, we prepared ss DNA fragments with a partial ds structure, based on the previous observation suggesting the involvement of HR in the gene correction process (*11*). The annealing of helper oligonucleotides increased the gene correction efficiencies, supporting our hypothesis that a key aspect of the enhanced gene correction was improved recognition by the RAD51 protein (Figure 1). Interestingly, the order of the gene correction efficiency was as follows: fragment D35 (5′-tailed duplex) > fragment A35 (3′-tailed duplex) > ss fragment. This agrees well with the order of in vitro strand exchange efficiency conducted by the RAD51 protein (*12*). This similarity also supports the idea that the RAD51 protein is an important player in gene correction.

We observed that the length of the helper ODNs affected the gene correction efficiency (Figure 2). The length of the ODN would affect the stability of the resulting duplex region of 5′-tailed DNA in cells. Another possible reason is the affinity for RAD51 (and/or other HR proteins). The RAD51 protein might bind tailed duplexes containing longer helper ODNs with higher affinity, resulting in more efficient gene correction. Alternatively, the length effects may be due to the longevity (or durability) of the ODNs in cells. In the case of longer ODNs, the duplex structure is expected to persist, even if subjected to partial digestion by exonucleases. The longevity of ODNs would depend on their length, and the intracellular stabilities of the ODNs determine those of the tailed duplexes.

The ENA 20mer improved the gene correction efficiency, as compared with the DNA 20mer (Figure 3). On the other hand, the BNA/LNA 20mer was not as effective as the ENA 20mer. The introduction of ENA and BNA/LNA residues increases the thermal stability of the duplex of the oligonucleotide with complementary DNA to a similar degree (*23, 24*). However, only the ENA 20mer outcompeted the DNA 20mer in terms of correction efficiency. Thus, the duplex stability could not explain the enhanced gene correction with the ENA oligonucleotide. It has been reported that BNA·DNA and ENA·DNA hybrids form an A-like duplex, but the conformations of both hybrids slightly differ from each other (*23, 26*). It was shown that the sugar−phosphate backbone (C5′−C4′−C3′−O3′) torsion angle δ of ENA is ~77° and that of BNA/LNA is ~66° (*23*). Typical δ torsion angle values of A-type DNA and RNA are 79° and 82°, respectively (*27*), suggesting that ENA has a sugar conformation similar to those of the native A-type nucleic acids. Thus, it is possible that RAD51 (or other HR proteins) might recognize the ENA·DNA hybrid with higher affinity than the BNA·DNA hybrid. Alternatively, the difference in the gene correction efficiency may be due to the longevity of ENA and BNA/LNA oligonucleotides in cells. Oligonucleotides modified with an ENA residue reportedly exhibit greater stability against exo- and endonucleases than those modified with a BNA/LNA residue (*23, 24*).

In contrast to the ENA oligonucleotide, the 2′-*O*-Me-RNA oligonucleotide did not increase the gene correction efficiency (Figure 3), although we expected that the formation of the A-form 2′-*O*-Me-RNA·DNA hybrid would enhance the recognition by the RAD51 protein. However, this was not the case. Instead, the annealing of the 2′-*O*-Me-RNA 20mer decreased the correction efficiency. This negative effect might be attributed to the inhibition of the binding to RAD51 and/or other HR proteins, and/or to a different disposition by binding to other intracellular protein(s).

We designed loop-forming ODNs to protect the ss DNA fragment from nucleases. These ODNs enhanced the gene correction efficiency to levels similar to those of the corresponding straight 35mer helper ODNs [2.6% for fragment A35 vs 2.7% for the 5′-loop and 3.7% for fragment D35 vs 3.7% for the 3′-loop (Figures 1 and 3)], although the region hybridized to the ss DNA fragment was 20 nucleotides long. The binding of the ss DNA fragment and the loop-forming ODNs was stabilized further by coaxial stacking of the extraordinarily stable loop−stem portion.

The Hyg−EGFP and *rpsL* genes were corrected by the 5′-tailed ds DNAs with different efficiencies (Figures 1 and 5). These results suggest that the gene correction efficiency with the 5′-tailed ds DNAs depended on sequence contexts. This interpretation is consistent with our recent finding that gene correction efficiencies with ss DNA fragments varied depending on the position of an identical target gene (H. Kamiya et al., unpublished results).

We used episomal genes as model target genes to examine the effects of oligonucleotides bound to the ss DNA fragment on gene correction efficiency. Quantitative data were obtained by counting *E. coli* colonies, since one copy of the plasmid DNA would be introduced upon transformation of the bacterial cells. Note that the gene correction does not occur in *E. coli*, because the direct introduction of the DNA fragment and pTENHEX into bacterial cells did not result in the formation of the corrected molecule (data not shown). In addition, the transfection efficiency would not be the absolute determinant for the correction efficiency, when the tailed DNA fragments are co-introduced with the target plasmid. However, genes in chromosomal DNA will be the targets in our goal of clinical application. Thus, the transfection efficiency would absolutely determine the correction efficiency in this case. Moreover, the status of the chromatin structure would affect the correction of a gene on a chromosome (*28*). The development of both an effective gene correction device as the tailed ds DNA fragments and an

efficient delivery system is the next step required for the clinical use of this technology. Recently, Urnov et al. reported highly efficient gene correction with a plasmid as a donor, after ds break induction by a zinc-finger nuclease (*29*). The tailed duplex used in this study may function as a better donor in this novel gene correction strategy.

In conclusion, we showed that the annealing of a helper oligonucleotide to an ss DNA fragment enhanced gene correction. This result suggests that gene correction using tailed ds DNA fragments will be an attractive strategy for personalized medicine based on an individual's genetic information.

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SUPPORTING INFORMATION AVAILABLE

Effects of the length of helper ODNs on the gene correction efficiency (Figure 1). This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Gruenert, D. C., Bruscia, E., Novelli, G., Colosimo, A., Dallapiccola, B., Sangiuolo, F., and Goncz, K. K. (2003) Sequence-specific modification of genomic DNA by small DNA fragments. *J. Clin. Invest. 112*, 637–641.
2. Sangiuolo, F., and Novelli, G. (2004) Sequence-specific modification of mouse genomic DNA mediated by gene targeting techniques. *Cytogenet. Genome Res. 105*, 435–441.
3. Richardson, P. D., Augustin, L. B., Kren, B. T., and Steer, C. J. (2002) Gene repair and transposon-mediated gene therapy. *Stem Cells 20*, 105–118.
4. de Semir, D., and Aran, J. M. (2006) Targeted gene repair: The ups and downs of a promising gene therapy approach. *Curr. Gene Ther. 6*, 481–504.
5. Bertoni, C. (2005) Oligonucleotide-mediated gene editing for neuromuscular disorders. *Acta Myol. 24*, 194–201.
6. Parekh-Olmedo, H., and Kmiec, E. B. (2007) Progress and prospects: Targeted gene alteration (TGA). *Gene Ther. 14*, 1675–1680.
7. Igoucheva, O., Alexeev, V., and Yoon, K. (2004) Oligonucleotide-directed mutagenesis and targeted gene correction: A mechanistic point of view. *Curr. Mol. Med. 4*, 445–463.
8. Seidman, M. M., and Glazer, P. M. (2003) The potential for gene repair via triple helix formation. *J. Clin. Invest. 112*, 487–494.
9. Andersen, M. S., Sorensen, C. B., Bolund, L., and Jensen, T. G. (2002) Mechanisms underlying targeted gene correction using chimeric RNA/DNA and single-stranded DNA oligonucleotides. *J. Mol. Med. 80*, 770–781.
10. Tsuchiya, H., Harashima, H., and Kamiya, H. (2005) Increased SFHR gene correction efficiency with sense single-stranded DNA. *J. Gene Med. 7*, 486–493.
11. Tsuchiya, H., Harashima, H., and Kamiya, H. (2005) Factors affecting SFHR gene correction efficiency with single-stranded DNA fragment. *Biochem. Biophys. Res. Commun. 336*, 1194–1200.
12. Mazin, A. V., Zaitseva, E., Sung, P., and Kowalczykowski, S. C. (2000) Tailed duplex DNA is the preferred substrate for Rad51 protein-mediated homologous pairing. *EMBO J. 19*, 1148–1156.
13. Egashira, A., Yamauchi, K., Yoshiyama, K., Kawate, H., Katsuki, M., Sekiguchi, M., Sugimachi, K., Maki, H., and Tsuzuki, T. (2002) Mutational specificity of mice defective in the MTH1 and/or the MSH2 genes. *DNA Repair 11*, 881–893.
14. Shibahara, S., Mukai, S., Nishihara, T., Inoue, H., Ohtsuka, E., and Morisawa, H. (1987) Site-directed cleavage of RNA. *Nucleic Acids Res. 15*, 4403–4415.
15. Inoue, H., Hayase, Y., Imura, A., Iwai, S., Miura, K., and Ohtsuka, E. (1987) Synthesis and hybridization studies on two complementary nona(2′-*O*-methyl)ribonucleotides. *Nucleic Acids Res. 15*, 6131–6148.
16. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning*, 3rd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.
17. Bottger, E. C. (1998) High-efficiency generation of plasmid cDNA libraries using electro-transformation. *BioTechniques 6*, 878–880.
18. Saenger, W. (1973) Structure and function of nucleosides and nucleotides. *Angew. Chem., Int. Ed. 12*, 591–601.
19. Nishinaka, T., Shinohara, A., Ito, Y., Yokoyama, S., and Shibata, T. (1998) Base pair switching by interconversion of sugar puckers in DNA extended by proteins of RecA-family: A model for homology search in homologous genetic recombination. *Proc. Natl. Acad. Sci. U.S.A. 95*, 11071–11076.
20. Blommers, M. J., Pieles, U., and De Mesmaeker, A. (1994) An approach to the structure determination of nucleic acid analogues hybridized to RNA. NMR studies of a duplex between 2′-*O*-Me RNA and an oligonucleotide containing a single amide backbone modification. *Nucleic Acids Res. 22*, 4187–4194.
21. Obika, S., Nanbu, D., Hari, Y., Morio, K., In, Y., Ishida, T., and Imanishi, T. (1997) Synthesis of 2′-*O*,4′-*C*-methyleneuridine and -cytidine. Novel bicyclic nucleosides having a fixed C3′-endo sugar puckering. *Tetrahedron Lett. 38*, 8735–8738.
22. Koshkin, A. A., Singh, S. K., Nielsen, P., Rajwanshi, V. K., Kumar, R., Meldgaard, M., Olsen, C. E., and Wengel, J. (1998) LNA (locked nucleic acids): Synthesis of the adenine, cytosine, guanine, 5-methylcytosine, thymine and uracil bicyclonucleoside monomers, oligomerisation, and unprecedented nucleic acid recognition. *Tetrahedron 54*, 3607–3630.
23. Morita, K., Takagi, M., Hasegawa, C., Kaneko, M., Tsutsumi, S., Sone, J., Ishikawa, T., Imanishi, T., and Koizumi, M. (2003) Synthesis and properties of 2′-*O*,4′-*C*-ethylene-bridged nucleic acids (ENA) as effective antisense oligonucleotides. *Bioorg. Med. Chem. 11*, 2211–2226.
24. Morita, K., Hasegawa, C., Kaneko, M., Tsutsumi, S., Sone, J., Ishikawa, T., Imanishi, T., and Koizumi, M. (2002) 2′-*O*,4′-*C*-Ethylene-bridged nucleic acids (ENA): Highly nuclease-resistant and thermodynamically stable oligonucleotides for antisense drug. *Bioorg. Med. Chem. Lett. 12*, 73–76.
25. Hirao, I., Nishimura, Y., Tagawa, Y., Watanabe, K., and Miura, K. (1992) Extraordinarily stable mini-hairpins: Electrophoretical and thermal properties of the various sequence variants of d(GC-GAAAGC) and their effect on DNA sequencing. *Nucleic Acids Res. 20*, 3891–3896.
26. Koizumi, M. (2004) 2′-*O*,4′-*C*-Ethylene-bridged nucleic acids (ENA™) as next-generation antisense and antigene agents. *Biol. Pharm. Bull. 27*, 453–456.
27. Saenger, W. (1984) *Principles of Nucleic Acid Structure*, Springer-Verlag, New York.
28. Cummings, W. J., Yabuki, M., Ordinario, E. C., Bednarski, D. W., Quay, S., and Maizels, N. (2007) Chromatin structure regulates gene conversion. *PLoS Biol. 5*, e246.
29. Urnov, F. D., Miller, J. C., Lee, Y. L., Beausejour, C. M., Rock, J. M., Augustus, S., Jamieson, A. C., Porteus, M. H., Gregory, P. D., and Holmes, M. C. (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature 435*, 646–651.

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