

Effects of Target Sequence and Sense versus Anti-sense Strands on Gene Correction with Single-stranded DNA Fragments

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The correction of an inactivated hygromycin resistance and enhanced green fluorescent protein (Hyg-EGFP) fusion gene by a several hundred-base single-stranded (ss) DNA fragment has been reported. In this study, the effectiveness of this type of gene correction was examined for various positions in the *rpsL* gene. Sense and anti-sense ssDNA fragments were prepared, and the gene correction efficiencies were determined by co-introduction of the target plasmid containing the gene with the ssDNA fragments. The gene correction efficiency varied (0.8–9.3%), depending on target positions and sense/anti-sense strands. Sense ssDNA fragments corrected the target gene with equal or higher efficiencies as compared to their anti-sense counterparts. The target positions corrected with high efficiency by the sense fragments also tended to be corrected efficiently by the anti-sense fragments. These results suggest that the sense ssDNA fragments are useful for the correction of mutated genes. The variation in the correction efficiency may depend on the sequence of the target position in double-stranded DNA.

Key words: gene correction, genetic engineering, nucleic acid therapeutics, *rpsL* gene, single-stranded DNA fragment.

Abbreviations: ds, double-stranded; EGFP, enhanced green fluorescent protein; Hyg, hygromycin-resistance; ss, single-stranded.

Gene correction (nucleotide sequence conversion), by which a mutated gene is converted to one with the normal (or desired) sequence, is an attractive strategy for gene therapy (1–10). Disruption of a gene involved in maintenance of a disease could be conducted with this technology. The corrected genes can be expressed under the control of their authentic regulatory elements. Moreover, gain-of-function or dominant mutations, such as activated oncogenes, could be suitable subjects for the gene correction strategy. Various kinds of devices, such as ss oligonucleotides, triplex-forming oligonucleotides and heat-denatured 400–800 bp double-stranded (ds) PCR fragments, have been examined as nucleic acid tools for gene correction (1–10).

Previously, it was reported that a several hundred-base single-stranded (ss) DNA fragment containing the sense sequence, prepared by restriction enzyme digestions of ss phagemid DNA, corrected an inactivated episomal hygromycin resistance and enhanced green fluorescent protein (Hyg-EGFP) fusion gene with higher gene correction efficiency, in comparison with the conventional PCR fragment (11). In contrast, the correction with the ssDNA fragment containing the anti-sense sequence was less efficient than that with the sense ssDNA fragment.

These results raised the questions of whether ssDNA fragments could be used to correct different target sequences and whether sense fragments are more efficient than the corresponding anti-sense fragments for other target sequences. In this study, we chose the *rpsL* (bacterial streptomycin-resistance) gene as the target gene. The gene encodes the S12 protein, one of the small subunit proteins of the *Escherichia coli* ribosome. Specific mutations at various positions in the *rpsL* gene confer streptomycin-resistant phenotype (*strA*) to bacteria. The *E. coli strA* strain harbouring the plasmid with the wild-type *rpsL* gene exhibits the streptomycin-sensitive phenotype, while the *strA* strain harbouring the plasmid with the mutated *rpsL* gene shows the streptomycin-resistant phenotype. Using the *rpsL* gene as the target, the gene correction efficiencies were examined at various positions within the gene. In addition, the efficiencies with sense and anti-sense ssDNA fragments were compared. The results obtained in this study indicate that the ssDNA fragment is useful for the correction of various target sequences, and that the sense fragments are equal or superior to the anti-sense fragments. Moreover, we found that the variation in the correction efficiency may depend on the sequence of the target position in the dsDNA.

MATERIALS AND METHODS

General—Oligodeoxyribonucleotides were purchased from Sigma Genosys Japan (Ishikari, Japan) and

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Invitrogen Japan (Tokyo, Japan) in purified forms. VCSM13 was from Stratagene (La Jolla, CA, USA). The *E. coli* strain DH10B (*strA* and *recA*) was from Takara (Kusatsu, Japan). Sequencing reactions were conducted with a BigDye Terminator v3.1 Cycle Sequencing kit and an ABI model 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The pSSW plasmid containing the wild-type *rpsL* gene and the kanamycin-resistance gene (*12*) were purified with a Qiagen (Hilden, Germany) EndoFree Plasmid Mega kit.

Plasmid and Phagemid Construction—The pSSW plasmid was introduced into MK602 (*mutT*) and MK611 (*mutM/mutY*) mutator *E. coli* strains (13). Spontaneous *rpsL* mutant colonies were randomly picked from streptomycin agar plates (described below), and the plasmid DNAs were isolated from the selected colonies. The small EcoRI–EcoRI fragments from the mutant plasmid DNAs were inserted into the large EcoRI–EcoRI fragment of pSVKam189 phagemid DNA (14) to obtain phagemid DNAs, in which the mutant *rpsL* fragment was inserted in either the sense or anti-sense direction (Fig. 1A).

In the phagemids thus obtained, pSVKam-*rpsL*-S and pSVKam-*rpsL*-AS, the mutant *rpsL* gene was inserted in opposite directions relative to each other (Fig. 1A). The ss circular phagemids generated by pSVKam-*rpsL*-S and

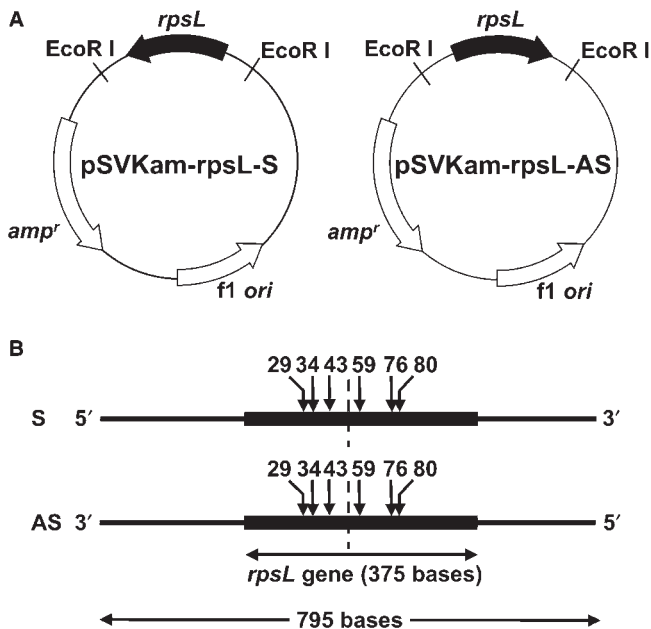


Fig. 1. Schematic representation of (A) the phagemid constructs and (B) the ssDNA fragments for the correction of the *rpsL* gene, used in this study. (A) The phagemids pSVKam-*rpsL*-S and pSVKam-*rpsL*-AS contain the EcoRI fragment encoding the *rpsL* gene in opposite directions. The ss forms of pSVKam-*rpsL*-S and pSVKam-*rpsL*-AS provide the sense and anti-sense ssDNA fragments, respectively, by EcoRI digestion. *amp^r*, *E. coli* ampicillin resistance gene; *f1 ori*, *f1* replication origin used for production of the ss phagemid DNAs. (B) The sense and anti-sense ssDNA fragments for the correction of six positions in the *rpsL* gene. The central positions of the fragments are indicated by vertical dashed lines. S and AS mean the sense and anti-sense DNA fragments, respectively.

pSVKam-*rpsL*-AS contain the sense and the anti-sense strand of the mutant *rpsL* gene, respectively. The insert directions were confirmed by digestion with BamHI and sequencing (data not shown). The *E. coli* JM105 cells harbouring each phagemid were cultured overnight in $2 \times$ YT medium containing 50 μ g/ml ampicillin. A 10 ml aliquot of the culture medium was transferred into 1 l of $2 \times$ YT medium (50 μ g/ml ampicillin) with an m.o.i. = 20:1 of VCSM13 helper phage, and the cells were then cultured with vigorous aeration at 37°C. Kanamycin was added into the culture medium at 1–1.5 h (final concentration 50–75 μ g/ml). The cultures were incubated further overnight, and then the phages were separated from the bacterial cells by centrifugation (2.15×10^3 g, 15 min). A 75 ml aliquot of 20% PEG 8000/2.5 M NaCl was added to each phage-containing supernatant, and the mixtures were incubated at 4°C overnight. The phages were precipitated by centrifugation (18.8×10^3 g, 20 min) and were resuspended in 20 ml of 0.3 M sodium acetate/1 mM EDTA. The ss phagemid was recovered by the standard phenol/chloroform extraction method. Each 10 ml DNA solution was mixed with 10 ml of isopropanol and placed at room temperature for 30 min. The ssDNA was precipitated by centrifugation (18.8×10^3 g, 20 min), and then was dissolved in 500 μ l of H₂O.

Preparation of DNA Fragments for Gene Correction—The 795-base ss fragments were prepared by annealing pSVKam-*rpsL*-S and pSVKam-*rpsL*-AS with their respective scaffold oligodeoxyribonucleotides (5'-dATCTTAAGA ATTCAGATCGAAG-3' and 5'-dAACC GGGAATTCAGCG TC-3' for pSVKam-*rpsL*-S, and 5'-dATATCTTAAGAATT CCCGGTTT-3' and 5'-dTCGATCTGAATTCAGCGTCA-3' for pSVKam-*rpsL*-AS, respectively) containing an EcoRI site (underlined), followed by EcoRI digestion (4.5 U/ μ g of ssDNA). The ssDNA fragments were purified by low-melting point agarose gel electrophoresis. The ssDNA fragments were further purified by gel filtration chromatography (NAP-5 column, GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Their UV spectra were measured to confirm their purities and to calculate their yields. The concentrations were determined by the molar absorption coefficient of DNA: 1.0 OD₂₆₀ equals 40 μ g of ssDNA (15).

Cell Culture and Transfection—CHO-K1 cells were maintained in DMEM/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and antibiotics, in a 5% CO₂ atmosphere at 37°C. One day before transfection, $3\text{--}5 \times 10^5$ cells, suspended in 4 ml of culture medium, were placed in a 6 cm dish.

The DNA fragments (10 pmol) were mixed with 25 fmol (50.7 ng) of pALTER-Ex2 (Promega), 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 μ g in this study. Transfection into CHO-K1 cells was carried out with the Lipofectamine Plus Reagent (Invitrogen), according to the supplier's instructions. At 48 h post-transfection, the cells were harvested, resuspended in 100 μ l of TEG (25 mM Tris-HCl, 10 mM EDTA, 50 mM glucose, pH 8.0) and slowly mixed with 200 μ l of 0.2 N NaOH/1% SDS. After the cell lysate had been incubated for 5 min

at room temperature, the lysate was neutralized with 150 μ l of 8M CH₃CONH₄, chilled at 4°C for 15 min and then centrifuged. The supernatant was transferred into a new tube, and the plasmid DNA was recovered by isopropanol precipitation and was dissolved in 10 μ l of H₂O.

Determination of Gene Correction Frequency—Electrocompetent *E. coli* DH10B cells were prepared essentially according to the method described in the literature (16). The electro-competent cells were mixed with the DNA recovered from the CHO-K1 cells and were electroporated at 4°C, 1.8 kV, 25 μ F and 200 Ω with a Gene Pulser II (Bio-Rad, Hercules, CA, USA). The cells were incubated with 1 ml of SOC medium for 1 h at 28°C. Dilutions (10- to 100-fold) of the cell suspension were seeded onto LB agar plates containing 200 μ g/ml of streptomycin and 50 μ g/ml of kanamycin (selection plates). Dilutions (1,000- to 10,000-fold) of the cell suspension were also seeded onto LB agar plates containing 50 μ g/ml of kanamycin (titer plates). The plates were incubated at 30°C, and the number of colonies on the selection plates was counted at 36–48 h. Gene correction frequencies were calculated by dividing the number of streptomycin-resistant colonies on the selection plates by the number of colonies on the titre plates.

RESULTS

Experimental Design—Sense and anti-sense ssDNA fragments reportedly corrected an inactivated Hyg-EGFP gene with a TGA sequence (termination codon) at codon 34 instead of TCA (Ser) (11). The sense ssDNA fragment corrected the Hyg-EGFP gene 9-fold more efficiently than the anti-sense counterpart. We examined whether these types of DNA fragments could correct other sequences in another target gene. We selected the *rpsL* gene as the target gene, since sequence conversions at various sites in the gene are detectable by a phenotypical change, from sensitivity to resistance to streptomycin (12). Previously, another antibiotic-resistance gene (the zeocin-resistance gene) was used as a target of gene correction with heat-denatured ds PCR fragments (10).

In this study, we examined gene correction at codons 29, 34, 43, 59, 76, and 80 (Table 1 and Fig. 1B).¹ At codon 29, correction of the CAA sequence to AAA (G:C→T:A) allows bacterial cells harbouring the corrected plasmid DNA to grow on agar plates containing streptomycin. Likewise, codons 34 and 76 are targets of a G:C→T:A correction. In addition, A:T→C:G corrections at codons 43, 59 and 80 are detectable by the phenotypical change (Table 1).

The *rpsL* gene with the desired sequence at the codons described above was inserted into the pSVKam189 phagemid DNA. The phagemids pSVKam-rpsL-S and pSVKam-rpsL-AS contain the entire *rpsL* gene, and each ss form carries the sense and anti-sense sequence of the gene, respectively (Fig. 1A). The 795-base ssDNA fragments were obtained by EcoRI digestion of ss pSVKam-rpsL-S and pSVKam-rpsL-AS, after annealing with

Table 1. Target sequences of gene correction.

Position	Sequence change ^a	
	Before	After
codon 29	cgc <u>CAA</u> aaa (Gln)	cgc <u>AAA</u> aaa (Lys)
codon 34	gta <u>TGT</u> act (Cys)	gta <u>TTT</u> act (Phe)
codon 43	cct <u>AAA</u> aaa (Lys)	cct <u>ACA</u> aaa (Thr)
codon 59	act <u>AAC</u> ggt (Asn)	act <u>CAC</u> ggt (His)
codon 76	cag <u>GAG</u> cac (Glu)	cag <u>TAG</u> cac (term)
codon 80	gtg <u>ATC</u> ctg (Ile)	gtg <u>AGC</u> ctg (Ser)

^aTarget codons and positions are capitalized and underlined, respectively. Adjacent sequences are also shown in lower-case letters. Amino acids corresponding the target codons are indicated in parentheses.

scaffold oligodeoxyribonucleotides complementary to the two EcoRI sites within the phagemid.

The pSSW plasmid carries the normal *rpsL* gene, and the *E. coli* cells harbouring this plasmid are sensitive to streptomycin. The pSSW plasmid, the gene correction target, was transfected into CHO-K1 cells together with the ssDNA fragment. After 48 h, the plasmid was isolated from the transfected cells, and the recovered plasmid DNA was then electroporated into *E. coli* DH10B cells. The bacterial cells become streptomycin resistant if the gene correction occurs. One copy of the plasmid DNA would be introduced upon transformation of the bacterial cells, and quantitative data could be obtained by counting the streptomycin-resistant *E. coli* colonies.

Gene Correction Efficiencies with ssDNA Fragments—We prepared the ssDNA fragments with sense and anti-sense sequences, and these ssDNA fragments (400-fold molar excess) were introduced into CHO-K1 cells together with the target plasmid, pSSW. The plasmids were recovered from the treated CHO-K1 cells, and then DH10B cells were transformed with the recovered plasmids. The gene correction efficiencies were calculated based on numbers of *E. coli* colonies on selection and titre agar plates. Total of more than 5,000 transformed *E. coli* cells were obtained by the electroporation when we determined the efficiencies. The gene correction efficiencies of the various ssDNA fragments are summarized in Fig. 2. All of the ssDNA fragments could correct the target gene, indicating that the gene correction with a several hundred-base ssDNA fragment is applicable for another gene and various target sequences. However, the efficiencies were variable, in position- and strand-dependent manners. For example, both the sense and anti-sense ssDNA fragments corrected codons 29 and 43 with ~1% efficiency (Fig. 2). On the other hand, the gene correction efficiency for codon 80 was ~9% with the sense ssDNA fragment, whereas that with the anti-sense fragment was ~3%, a 3-fold lower efficiency. Nearly, a 12-fold difference was observed in the gene correction efficiency (0.8% at codon 29 with the sense and anti-sense fragments and 9.3% at codon 80 with the sense fragment). The sense and anti-sense ssDNA fragments corrected with similar efficiencies at codons 29, 34, 43 and 76, and the sense DNA fragments tended to correct more efficiently than their anti-sense counterparts at codons 59 and 80 (Fig. 2).

¹ These target sites were determined by sequencing of streptomycin-resistant bacterial colonies spontaneously generated from mutator strains (*mutM/mutY* and *mutT*, 13).

The gene correction judged by the phenotypical change was confirmed by sequence analysis of the *rpsL* genes in the streptomycin-resistant bacterial colonies (Fig. 3). Therefore, the several hundred-base ssDNA fragment was an excellent gene correction tool.

We electroporated the pSSW plasmid and ssDNA fragment plus pSSW (400:1 molar ratio) into *E. coli* DH10B cells. The direct introduction of the former and the latter resulted in formation of the streptomycin-resistant *E. coli* colonies with frequencies of 0.0029% and 0.0079%, respectively (data not shown). These background levels were much lower than the gene correction efficiencies obtained with the ssDNA fragments used in this study. Thus, the efficiencies seemed to reflect the gene correction event(s) in CHO-K1 cells.

Factors Affecting ssDNA Fragment-mediated Gene Correction Efficiency—We then analysed the obtained gene correction efficiencies from various viewpoints. First, we analysed the strand bias of the gene correction

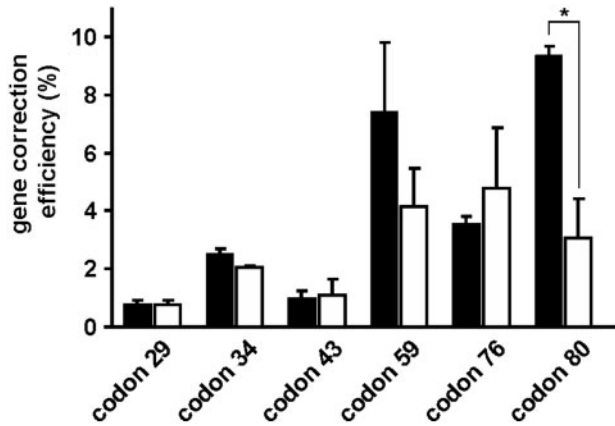


Fig. 2. **Gene correction efficiency with ssDNA fragments.** The DNA fragments were co-transfected with the target plasmid pSSW (400:1 molar ratio) into CHO-K1 cells, and the plasmids recovered from the cell at 48 h post-transfection were used to transform the *E. coli* DH10B strain. The numbers of colonies on agar plates with and without streptomycin were counted to determine the gene correction efficiencies. Closed columns, sense DNA fragment; open columns, anti-sense DNA fragment. Bars indicate SD ($n=3$) ($*P<0.05$).

efficiencies. Previously, the sense DNA fragment corrected the Hyg-EGFP gene with 9-fold more efficiency than the anti-sense fragment (11). As shown in Fig. 2, the gene correction efficiencies with the sense fragments were equal or superior to those with the anti-sense fragments. In the case of codon 80, the sense DNA fragment corrected the gene with 3-fold higher efficiency than the anti-sense fragment, as described above. Thus, the results of this and previous studies suggest the usefulness of sense ssDNA fragments.

Next, we focused on the type of sequence change at the target positions. Previously, the integration of the ssDNA fragment into the ds target plasmid DNA was observed (17). Thus, a mismatch base pair would be formed between the 'ssDNA fragment' and the complementary strand in the target DNA. The type of sequence change (i.e. mismatch pair) could affect the gene correction efficiency. We classified the changes into four groups, based on the assumption of the direct replacement of the target strand by the DNA fragment, focusing on the changes in the strand corresponding to the ssDNA fragments used (C→A, G→T, A→C and T→G, Fig. 4). For example, the change from CAA (Gln) to AAA (Lys) at codon 29 (Table 1) with sense and anti-sense DNA

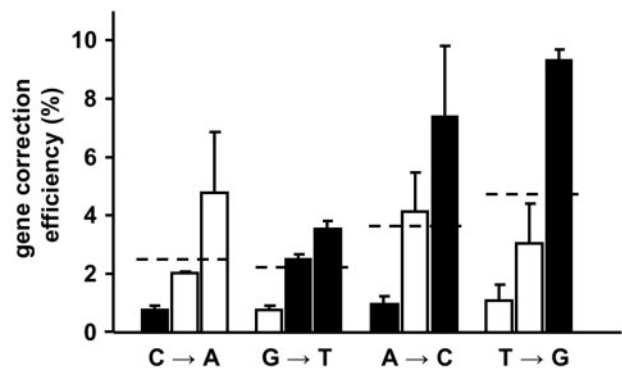


Fig. 4. **Comparison of the gene correction efficiency with ssDNA fragments.** The data shown in Fig. 2 are categorized by base changes. Closed columns, sense DNA fragment; open columns, anti-sense DNA fragment. Averages are shown by horizontal dashed lines.

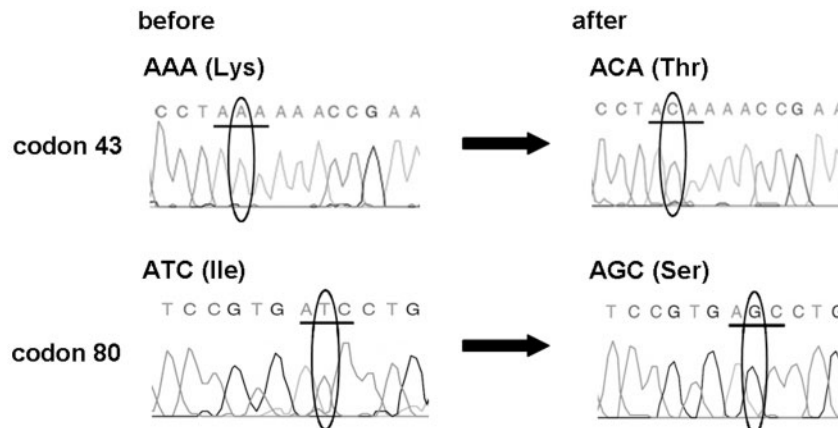


Fig. 3. **Confirmation of gene correction by sequencing of plasmids recovered from streptomycin-resistant colonies.** The results from two colonies are shown as an example. Bars indicate codons 43 and 80.

fragments were included in the C→A and G→T groups, respectively. All groups contained three independent sources of correction data, and their averages were calculated (shown by horizontal dashed lines). We did not observe a marked difference in the correction efficiency (C→A; $2.5 \pm 2.1\%$, G→T; $2.3 \pm 1.4\%$, A→C; $3.8 \pm 3.3\%$, T→G; $4.8 \pm 4.1\%$, where the values after \pm represent SD). Thus, the type of sequence change seemed to affect the gene correction efficiency only weakly, at least for the changes examined in this study. In addition, we did not find correlation between the gene correction efficiency and the sequence around the target positions (Table 1).

We then verified the position effects of the target bases on the correction efficiency. As shown in Fig. 2, the efficiencies seemed to be higher at codons 59, 76 and 80 than those at codons 29, 34 and 43, with both the sense and anti-sense DNA fragments. Indeed, the mean of the correction efficiencies by the sense fragments at codons 59, 76 and 80 was significantly higher than that at codons 29, 34 and 43 (6.7 versus 1.4%, Table 2). Likewise, the mean of the correction efficiencies by the anti-sense fragments at the former codons was significantly higher than that at the latter codons (Table 2). Codons 59, 76 and 80 were located in the 3'- and 5'-regions in the sense and anti-sense DNA fragments, respectively (Fig. 1B). Thus, neither the 5'- nor 3'-region was superior for the correction, when we did not discriminate between the sense or anti-sense strand. We then interpreted that codons 59, 76 and 80 were located in the 'downstream' region of the target dsDNA. Next, we calculated the average correction efficiencies for the upstream (codons 29, 34 and 43) and downstream (codons 59, 76 and 80) regions, without discrimination between the sense and anti-sense ss fragments. A statistically significant difference existed between the former and latter efficiencies (Table 2). Thus, the gene correction efficiency may be highly dependent on the sequence of the target dsDNA. In addition, this result may suggest that the target positions that were quite efficiently corrected by the sense fragments also tended to be efficiently corrected by the anti-sense fragments.

DISCUSSION

One of the major objectives of this study was to examine whether ssDNA fragments could correct various sequences in a new target gene, the *rpsL* gene. As shown in Fig. 2, ssDNA fragments corrected various sequences in the gene, although the correction efficiencies varied, from 0.8% to 9.3%, depending on the target positions. Nearly, a 12-fold difference was observed when six positions in the same gene were correction targets. When we classified the sequence changes into four groups (C→A, G→T, A→C and T→G) by the changes in the strand corresponding to the ssDNA fragments used, no tendency was apparent for the type of sequence change (*i.e.* mismatch base pair) at least with the four cases examined in this study (Fig. 4). Thus, the type of sequence change could not explain the one order of magnitude difference in the correction efficiency. On the other hand, we found a similar tendency between the gene correction efficiency with the sense ssDNA

Table 2. Variation in gene correction efficiency.

DNA fragment	Gene correction efficiency (%)		<i>P</i>
	Upstream (codons 29, 34, 43)	Downstream (codons 59, 76, 80)	
Sense	1.4 ± 0.9	6.7 ± 2.9	<0.05
Anti-sense	1.3 ± 0.7	4.0 ± 0.9	<0.05
Sense/anti-sense	1.4 ± 0.7	5.4 ± 2.5	<0.01

Data are expressed as mean \pm SD.

fragments and that with the anti-sense DNA fragments. Statistical significance was found in the gene correction efficiency for the upstream (codons 29, 34 and 43) and downstream (codons 59, 76 and 80) regions with the sense, anti-sense and sense plus anti-sense DNA fragments (Table 2). This result suggests that the variation in the correction efficiency with either the sense or anti-sense fragments may be highly dependent on the sequence of the target dsDNA. Previously, the possibility that the introduced ssDNA fragment was integrated into the target plasmid DNA was shown (17). Thus, homologous recombination may be involved, at least in part, in the gene correction process with the ssDNA fragment. From this viewpoint, the sequence-dependent recognition efficiency of the gene correction target by the protein(s) involved in homologous recombination may be a determinant factor for the correction efficiency. We observed variety of gene correction efficiencies (Fig. 2). We correlated this result with the sequence of the target dsDNA, instead of the target position and the target plus surrounding sequences in the ssDNA fragments. However, the DNA fragments used in this study were not ideal to evaluate the factors since the position and the target plus surrounding sequences were not identical. Further study will draw the final conclusion for the reason(s) of the various gene correction efficiencies.

The other major objective of this study was to examine the effects of the strand of the ssDNA fragments. In the previous study using the Hyg-EGFP gene, a sense ssDNA fragment corrected the target gene 9-fold more efficiently than the corresponding anti-sense ssDNA fragment (11). In the case of the *rpsL* gene examined in this study, similar gene correction efficiencies were observed at four positions, and more efficient correction with the sense fragments was found at codons 59 and 80 (Fig. 2). In contrast to the case of the Hyg-EGFP gene, only a 3-fold difference was seen at codon 80 (9.3% versus 3.1%). Taken together, we concluded that the sense ssDNA fragments were at least equal to and sometimes superior to the anti-sense fragments.

This strand bias found in some cases could not be explained by transcription. Plasmid DNA lacking a mammalian promoter was used in this study, and the gene correction efficiencies by the sense and anti-sense DNA fragments for the promoterless Hyg-EGFP plasmid DNA were as efficient as that with the Hyg-EGFP plasmid containing a mammalian promoter (17). Additionally, the strand bias was not due to replication (lagging versus leading strand syntheses), since the target plasmids lack a mammalian origin of replication. It has been shown that the strand bias of gene correction by ss oligonucleotide in mammalian cells was affected by

various factors (18–21). It will be important to determine the actual reason(s) for the strand bias of gene correction by the longer ssDNA fragments used in this study.

We previously found that the ssDNA fragment was integrated into the ds target DNA, suggesting that homologous recombination was involved, at least in part, in the gene correction process with the ssDNA fragment (17). The ssDNA fragment might invade the ds plasmid to form the ‘D-loop’ structure by protein(s) involved in homologous recombination, followed by strand replacement and subsequent removal of the target base by mismatch repair (fixation). We found the possibility that the gene correction efficiency is highly dependent on the sequence of the target dsDNA. This dependency might be related to the putative strand invasion, strand replacement and/or removal of the target base.

We recovered the plasmid DNA from the CHO-K1 cells. The ssDNA fragment co-transfected with the plasmid DNA might be included in the recovered DNA fraction. Because the ssDNA fragments seem to be substrates of exonucleases in addition to endonucleases, they would be less stable in cells than the circular plasmid DNA that would not be degraded by exonucleases. Thus, the ratio of ssDNA fragment to the plasmid seemed to be less than 400:1 in the recovered DNA fraction. The direct introduction of ssDNA fragment and pSSW (400:1 molar ratio) into *E. coli* DH10B cells resulted in ‘gene correction’ with frequency of 0.0079%, much less frequency (data not shown). Thus, the efficiencies shown in Fig. 2 seemed to reflect the gene correction event(s) in CHO-K1 cells. This may be explained by the fact that DH10B is a strain deficient in RecA, a protein involved in homologous recombination in *E. coli*.

In this study, we used the episomal *rpsL* gene as a model target gene. The ssDNA fragment was co-introduced with the target *rpsL* plasmid, and transfection efficiency would not be the absolute determinant for the correction efficiency. On the other hand, the introduction efficiency would absolutely determine the correction efficiency in the case of genes in chromosomal DNA. In addition, the status of the chromatin structure would affect the correction of genes on chromosomes (22). The development of an efficient delivery system that also controls intracellular and intranuclear dispositions of ssDNA fragments is required for the clinical use.

In conclusion, we showed that ssDNA fragments could correct various sequences and would be useful in the correction of mutated genes. These results suggest that gene correction using this type of ssDNA fragment, with further improvements, will be an attractive strategy for personalized medicine based on an individual’s genetic information.

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REFERENCES

- 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
- Sanguolo, F. and Novelli, G. (2004) Sequence-specific modification of mouse genomic DNA mediated by gene targeting techniques. *Cytogenet. Genome Res.* **105**, 435–441
- 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
- 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
- Bertoni, C. (2005) Oligonucleotide-mediated gene editing for neuromuscular disorders. *Acta Myol.* **24**, 194–201
- Parekh-Olmedo, H., Ferrara, L., Brachman, E., and Kmiec, E.B. (2005) Gene therapy progress and prospects: targeted gene repair. *Gene Ther.* **12**, 639–646
- 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
- Seidman, M.M. and Glazer, P.M. (2003) The potential for gene repair via triple helix formation. *J. Clin. Invest.* **112**, 487–494
- 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
- Colosimo, A., Goncz, K.K., Novelli, G., Dallapiccola, B., and Gruenert, D.C. (2001) Targeted correction of a defective selectable marker gene in human epithelial cells by small DNA fragments. *Mol. Ther.* **3**, 178–185
- Tsuchiya, H., Harashima, H., and Kamiya, H. (2005) Increased SFHR gene correction efficiency with sense single-stranded DNA. *J. Gene Med.* **7**, 486–493
- Egashira, A., Yamauchi, K., Yoshizawa, 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
- Tajiri, T., Maki, H., and Sekiguchi, M. (1995) Functional cooperation of MutT, MutM and MutY proteins in preventing mutations caused by spontaneous oxidation of guanine nucleotide in *Escherichia coli*. *Mutat. Res.* **336**, 257–267
- Kamiya, H. and Kasai, H. (2000) 2-Hydroxy-dATP is incorporated opposite G by *Escherichia coli* DNA polymerase III resulting in high mutagenicity. *Nucleic Acids Res.* **28**, 1640–1646
- Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning*, 3rd edn, Cold Spring Harbor Laboratory Press, New York
- Bottger, E.C. (1998) High-efficiency generation of plasmid cDNA libraries using electro-transformation. *Biotechniques* **6**, 878–880
- 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
- Brachman, E.E. and Kmiec, E.B. (2004) DNA replication and transcription direct a DNA strand bias in the process of targeted gene repair in mammalian cells. *J. Cell Sci.* **117**, 3867–3874
- Suzuki, T., Murai, A., and Muramatsu, T. (2005) Bleomycin stimulates targeted gene repair directed by single-stranded oligodeoxynucleotides in BHK-21 cells. *Int. J. Mol. Med.* **16**, 615–620
- Hu, Y., Parekh-Olmedo, H., Drury, M., Skogen, M., and Kmiec, E.B. (2005) Reaction parameters of targeted gene repair in mammalian cells. *Mol. Biotechnol.* **29**, 197–210
- Bertoni, C., Morris, G.E., and Rando, T.A. (2005) Strand bias in oligonucleotide-mediated dystrophin gene editing. *Hum. Mol. Genet.* **14**, 221–233
- 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