Correction of Frameshift Mutations with Tailed Duplex DNAs

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Tailed duplex (TD) DNAs, prepared by annealing an oligonucleotide to a several-hundred-base single-stranded (ss) DNA fragment, correct a base-substitution mutation with high efficiency. In the present study, the abilities of TD fragments to correct single-base insertion and deletion mutations were examined, using hygromycin-resistance and enhanced green fluorescent protein fusion (Hyg-EGFP) genes inactivated by +G and −C frameshift mutations. The 5′-TD and 3′-TD DNA fragments were co-transfected with plasmid DNA containing the inactivated Hyg-EGFP gene into CHO-K1 cells, and the gene correction efficiencies were determined by introducing the plasmid DNA recovered from the transfected cells into *Escherichia coli* cells. In contrast to their efficiencies for the substitution mutation, the gene correction abilities of the TD fragments were relatively low. The correction efficiencies by the TD fragments were apparently higher than that by a ss DNA fragment, one of the DNA fragments employed for gene correction. These results suggest that the TD fragments have the potential to correct frameshift mutations, although further improvement is required.

Key words gene correction; tailed duplex DNA fragment; frameshift mutation

Many mutations have been found in disease-responsible genes. Frameshift mutations alter the reading frame, thus inducing a completely different translation from the original one, and often inactivate genes by producing truncated, nonfunctional proteins, resulting in the onset of diseases. For example, Duchenne muscular dystrophy is caused by mutations, mainly frameshifts, in the dystrophin (*DMD*) gene.[1] Thus, frameshift mutations are interesting targets for gene correction, conversion of a mutated sequence to the normal one.

Chemically synthesized oligonucleotides and polymerase chain reaction (PCR) fragments have been used as nucleic acids for gene correction.[2—7] We previously reported that a single-stranded (ss) DNA fragment containing the sense sequence, and tailed duplex (TD) DNA fragments prepared by annealing an oligonucleotide to the ss DNA fragment, have the ability to correct single-base substitution mutations.[8—10] However, the ss DNA fragment corrects frameshift (single-base deletion and single-base insertion) mutations with low efficiencies.[11] Since the correction efficiencies of single-base substitution mutations by the TD fragments are higher than those by the ss DNA fragment,[10] it is important to examine the ability of the TD fragments to correct frameshift mutations.

In this study, we determined the efficiency of frameshift mutation correction by the TD DNA fragments. Episomal hygromycin-resistance (Hyg) and enhanced green fluorescent protein (EGFP) fusion genes, inactivated by single-base deletion and insertion mutations, were chosen as model targets. The TD fragments were co-introduced into CHO-K1 cells with a plasmid DNA carrying the target gene. The gene correction efficiency was quantitatively determined by counting the EGFP-positive and hygromycin-resistant *Escherichia coli* colonies, after recovering the plasmid DNA from the transfected cells and introducing it into bacterial cells. The results obtained in this study suggested that the TD fragments have the potential to correct frameshift mutations, although further improvements are necessary for their clinical use.

MATERIALS AND METHODS

General The pTENHEins, pTENHEdel, and pTENHEX plasmids and the pBSHES/Sense phagemid were the same DNAs described in our previous studies.[8,11] The plasmid DNAs were amplified in *E. coli* strain DH5α, and were purified with a Qiagen (Hilden, Germany) Endofree Plasmid Mega kit. Oligodeoxyribonucleotides were purchased from Sigma Genosys Japan (Ishikari, Japan) and Hokkaido System Science (Sapporo, Japan) in purified forms. Helper phage VCSM13 and *E. coli* strain BL21(DE3) were from Agilent Technologies (Santa Clara, CA, U.S.A.).

Preparation of DNA Fragments for Gene Correction The 606-base ss sense fragment was prepared from the ss pBSHES/Sense phagemid DNA, by annealing with its respective scaffold oligodeoxyribonucleotides followed by *Xho*I digestion, as described previously.[8] 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.[12] When used as the ss DNA fragment, it was heat-denatured at 98 °C for 5 min, and then was immediately chilled on ice for at least 5 min until transfection.

For the preparation of the TD fragments, the ss DNA fragment was mixed with a 10-fold molar excess of a 35mer oligonucleotide (5′-dGGTGCCGGACTTCGGGGCAGTCCTCGGCCCAAAGC-3′ for 5′-TD and 5′-dCTTTTTCATGGTGGCGGCGGCTCCGGGGATCTCGA-3′ for 3′-TD).[10] 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 CHO-K1 cells were maintained in Dulbecco's modified Eagle's medium

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(DMEM)/F12 (Life Technologies, Carlsbad, CA, U.S.A.) medium supplemented with 10% fetal bovine serum and antibiotics, in a 5% CO_2 atmosphere at 37 °C. One day before transfection, 3×10^5 cells, suspended in 4 ml of culture medium, were placed in a 6-cm dish.

The DNA fragment (10 pmol) was mixed with 125 ng (25 fmol) of a target plasmid (pTENHEins, pTENHEdel, and pTENHEX). An appropriate amount of pALTER-Ex2 (Promega, Madison, WI, U.S.A.), which does not affect the gene correction assay, was added to bring the total amount of DNA to 4 μg. Transfection into CHO-K1 cells was performed with the Lipofectamine Plus Reagent (Life Technologies), according to the supplier's instructions. After 48 h, the cells were harvested, and the plasmid DNA was recovered, as described previously.[8)]

Determination of Gene Correction Efficiency and Genotypic Analysis Electro-competent *E. coli* cells were prepared essentially according to the method described in the literature.[13)] The plasmid DNA recovered from CHO-K1 cells was introduced into electro-competent DH5α cells, and the amplified plasmid DNA was isolated as described previously.[8)] A 2-μl aliquot of the plasmid DNA solution, recovered from the transformed DH5α cells, was electroporated into BL21(DE3) cells, which were incubated in 1 ml of SOC medium at 37 °C for 1 h. The bacterial cells were transferred into 9 ml of LB medium, containing 50 μg/ml of ampicillin and 10 μM of isopropyl-β-D-thiogalactopyranoside (IPTG), and the cells were further incubated for 3 h. Dilutions (10—10^4-fold) of the cell suspension were seeded onto LB agar plates containing 50 μg/ml of ampicillin, 10 μM of IPTG, and 50 μg/ml of hygromycin (the +Hyg plates). Dilutions (10^2—10^7-fold) of the cell suspension were also seeded onto LB agar plates containing 50 μg/ml of ampicillin and 10 μM of IPTG (the titer plates). The plates were incubated at 37 °C, and the number of colonies on the titer plates was counted at 12 h. Since a small fraction of bacterial cells carrying an inactivated Hyg-EGFP gene appeared on the +Hyg plate, these false-positive colonies were excluded from the calculation, by an analysis with a Fuji FLA2000G image analyzer (Tokyo, Japan) at 36 h after transformation. Gene correction efficiencies were calculated by dividing the number of EGFP-positive colonies on the +Hyg plates by the number of colonies on the titer plates. Under our conditions, we obtained $>10^6$ bacterial colonies on the titer plates.

Amplification and sequencing of the region containing the target position were conducted using the primers (5′-dTAATACGACTCACTATAGGG-3′ and 5′-dATCGCCTCGCTCCAGTCAAT-3′), as described previously.[8)]

RESULTS

Correction of Frameshift Mutations by Tailed Duplex DNA Fragments We previously examined the correction of a C to G substitution mutation by TD DNA fragments with a mutated Hyg-EGFP gene, in which codon 34 is TGA (termination codon), instead of the normal TCA (Ser) sequence, and found that the 5′-TD fragment corrected the mutation more efficiently than the ss and 3′-TD fragments (Fig. 1).[10)] To compare the efficiencies of +G and −C frameshift mutation correction by these DNA fragments, plasmid DNAs with the mutated Hyg-EGFP gene were used.

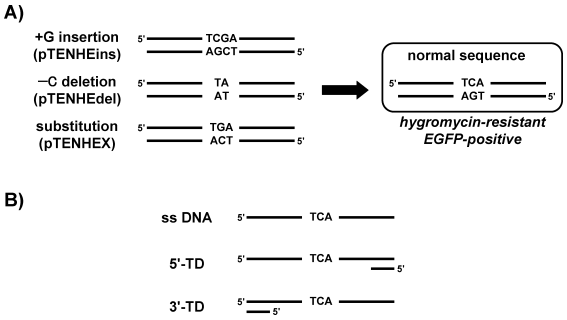

Fig. 1. Schematic Presentation of (A) the Hyg-EGFP Genes on the Various Plasmids Used in This Study and (B) the DNA Fragments for Gene Correction

(A) The Hyg-EGFP genes containing +G insertion and −C deletion mutations at codon 34 are shown. pTENHEX was used for comparison. The normal sequence is also shown. (B) The DNA fragments for gene correction are shown with the sequences corresponding to codon 34. The T base of the TCA sequence is the 126th base from the 5′-terminus.

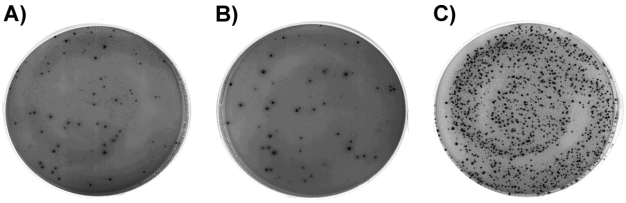

Fig. 2. EGFP-Expressing *E. coli* Colonies on Hygromycin Plates

The 5′-TD DNA fragment was co-transfected with the target plasmids, (A) pTENHEins (+G insertion), (B) pTENHEdel (−C deletion), and (C) pTENHEX (C to G substitution), at a 400 : 1 molar ratio into CHO-K1 cells, and the plasmids recovered from the cells at 48-h posttransfection were used to transform the BL21(DE3) cells. The colonies expressing EGFP were analyzed with an image analyzer. The black dots on the plates indicate the EGFP-positive colonies.

As in the previous study,[10)] we introduced the ss DNA fragment with the sense sequence, along with the 5′-TD and 3′-TD fragments, into CHO-K1 cells together with a target plasmid (Fig. 1B). Each DNA fragment was used in a 400-fold molar excess over the target plasmid, and the DNAs were transfected with cationic lipids. After 48 h, the plasmid DNA was recovered from the transfected CHO-K1 cells and introduced into *E. coli*. The transformed *E. coli* BL21(DE3) cells were seeded on agar plates containing hygromycin (+Hyg). The number of EGFP-positive colonies on the +Hyg plates was counted with a fluorescence image analyzer (dual phenotypic selection) to ensure the precise estimation of the gene correction efficiencies, by excluding the false positive colonies that appeared on the plates. The correction efficiencies were calculated using the number of hygromycin-resistant and EGFP-positive colonies, as described in Materials and Methods.

We first examined the correction of the +G insertion mutation with the ss and TD DNA fragments. As shown in Figs. 2A and C, the correction efficiency of the insertion mutation was quite low, as compared to that of the substitution mutation. The ss DNA fragment converted the insertion mutation to the normal sequence with an efficiency of 1.1×10^{-4} (Fig. 3A). The 5′- and 3′-TD DNA fragments seemed more efficient (2.2 and 2.9×10^{-4}, respectively) than the ss DNA fragment, although the difference was statistically insignificant. Thus, the TD fragments showed two- to three-fold improved correction efficiencies, as compared to the ss DNA fragment.

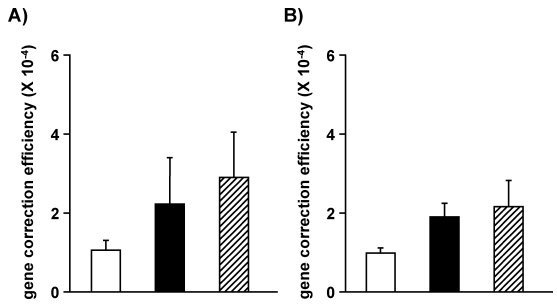

Fig. 3. Correction Efficiencies of (A) +G Insertion and (B) −C Deletion Mutations with the TD DNA Fragments

The ss and TD DNA fragments were co-transfected with the target plasmids, pTENHEins (+G insertion) and pTENHEdel (−C deletion), at a 400 : 1 molar ratio into CHO-K1 cells, and the plasmids recovered from the cells at 48-h posttransfection were used to transform the BL21(DE3) cells. Gene correction efficiencies were determined as described in Materials and Methods. The values represent the averages of three separate experiments. White columns, ss DNA fragment; black columns, 5′-TD fragment; hatched columns, 3′-TD fragment. Bars indicate SEM.

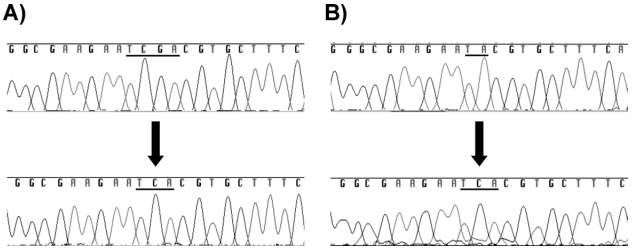

Fig. 4. Genotypic Analysis of EGFP-Expressing Colonies

Corrections of (A) the +G insertion and (B) the −C deletion mutations were confirmed by sequencing the plasmid DNAs recovered from EGFP-positive, hygromycin-resistant *E. coli* colonies. Sequences of plasmids obtained with the 5′-TD fragment are shown.

Next, we examined the correction of the deletion mutation. Similar results were observed in the experiments with the pTENHEdel plasmid DNA containing the −C deletion mutation (Figs. 2B, 3B). The ss DNA fragment corrected the deletion with an efficiency of 1.0×10^{-4}. In contrast, the TD fragments showed approximately two-fold improved correction efficiencies. Again, the TD fragments seemed more effective than the ss DNA fragment in the correction of the deletion mutation.

When the plasmid DNA containing the single-base substitution mutation (TGA) was tested, the 5′-TD DNA fragment corrected the Hyg-EGFP gene with an efficiency of 5.9×10^{-3} (Fig. 2C and data not shown). This value was *ca.* 20-fold higher than the correction efficiencies for the insertion and deletion mutations.

No EGFP-positive and hygromycin-resistant colonies were formed when the target plasmid and the TD fragment were directly introduced into *E. coli* cells (data not shown). Thus, the gene correction only occurs in the transfected CHO-K1 cells. In addition, the "gene correction" efficiencies were less than 4.0×10^{-6} when the target plasmid DNAs containing the +G and −C mutations were transfected into CHO-K1 cells without the DNA fragments (data not shown). Therefore, the TD DNA fragments had the ability to correct the +1 and −1 frameshift mutations, but their efficiencies were much lower than that for the single-base substitution mutation.

Sequence-Specificity of TD Fragment-Mediated Gene Correction The gene correction judged by the phenotypical change was confirmed by a sequence analysis of the Hyg-EGFP genes in EGFP-positive and hygromycin-resistant bacterial colonies (total 25 colonies). As shown in Fig. 4, the successful correction at the targeted position was confirmed, and no nucleotide alterations in the flanking sequences were found. Thus, the TD DNA fragments could correct the frameshift mutations without inducing mutations at other sites.

DISCUSSION

The objective of this study was to examine the efficiencies of frameshift mutation correction by the TD DNA fragments. In the case of a single-base substitution mutation, the 5′-TD DNA fragment corrects the mutation quite efficiently, as compared to the ss and 3′-TD DNA fragments.[10] In addition, the 3′-TD fragment corrects the substitution with slightly but significantly higher efficiency than the ss DNA fragment. As shown in Fig. 3A, the TD fragments seemed to exhibit higher gene correction ability for the +G mutation than the ss DNA fragment. However, no advantage of the 5′-TD DNA fragment over the 3′-TD fragment was observed, in contrast to the correction of the single-base substitution mutation. Likewise, in the case of the −C frameshift mutation, the TD fragments seemed to correct it more effectively than the ss DNA fragment, and the two types of TD fragments had comparable abilities (Fig. 3B). The correction efficiencies of the +G and −C frameshift mutations were similar for both fragments (Fig. 3). The results shown here suggested that the TD fragments have the potential to correct frameshift mutations.

However, the correction efficiencies of the +G and −C frameshift mutations with the TD fragments were one order of magnitude lower than those of the substitution mutation. Homologous recombination (HR), in which the pairing process is crucially important, seems to be involved in the gene correction process. For example, we previously showed that an introduced radioactive ss DNA fragment was incorporated into the target plasmid DNA in CHO-K1 cells.[14] Likewise, the radioactive TD fragment was also integrated into the target DNA (Tsuchiya *et al.*, unpublished results). In addition, the order of the correction efficiencies for the substitution mutation (5′-TD>3′-TD>ss DNA fragment) was identical to that of the *in vitro* strand exchange efficiencies conducted by the RAD51 protein, an important enzyme in HR.[15] Thus, the low gene correction efficiencies for the frameshift mutations, observed in this study, may be explained by the lower strand exchange efficiencies by the HR-related proteins in cells. Holmes *et al.* reported that *in vitro* strand transfer reactions conducted by the yeast Rad51 protein decreased when "frameshift mutations" were present.[16] Using the *E. coli* RecA protein, the bacterial counterpart of RAD51, Bucka and Stasiak demonstrated that *in vitro* strand exchange occurred in the absence of ATP hydrolysis for "substitution mutations," while ATP hydrolysis was required for "frameshift mutations."[17] They speculated that the rearrangement of the RecA filament, accompanied by ATP hydrolysis, was necessary for the strand exchange reactions containing the frameshift mutations. Thus, the lower gene correction efficiencies for the frameshift mutations were attributable to the similar preference of the mammalian RAD51 protein. However, the possibility that the frameshift mutation correction proceeded by pathway(s) other than HR

cannot be disregarded at this time.

In conclusion, the TD fragments have the potential to correct frameshift mutations, but their efficiencies are low. Thus, further enhancement of the correction efficiency by fragment modifications is necessary. Studies toward this goal are now in progress.

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