Correction of Frameshift Mutations with Single-Stranded and Double-Stranded DNA Fragments Prepared from Phagemid/Plasmid DNAs

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We recently found that a heat-denatured, double-stranded DNA fragment, prepared from plasmid DNA (dsHES), and a sense single-stranded DNA fragment, prepared from single-stranded phagemid DNA (fSense), corrected an inactivated hygromycin-resistance and enhanced green fluorescence protein fusion (Hyg-EGFP) gene containing a base substitution (G:C to C:G) mutation 2-fold and more than 10-fold, respectively, more efficiently than the conventional PCR fragment (pcrHES), in the small fragment homologous replacement method. In this study, we tested the abilities of these new DNA fragments to correct Hyg-EGFP genes inactivated by one base insertion (+G) and deletion (−C) mutations. In contrast to its activity with the substitution mutation, the fSense fragment showed similar efficiencies to those of the dsHES fragment in the correction of frameshift mutations. For the correction of the insertion mutation, the efficiencies were in the order of dsHES (0.21%) > fSense (0.18%) > pcrHES (0.08%). In the case of the correction of the deletion mutation, the efficiencies were in the order of fSense (0.27%) > dsHES (0.19%) > pcrHES (0.12%). These results suggest that sense single- and double-stranded DNA fragments prepared from phagemid and plasmid DNAs, respectively, have the potential to correct frameshift mutations.

Key words gene correction; small fragment homologous replacement (SFHR); frameshift mutation; single-stranded DNA fragment

Heat-denatured, 400—800 bp double-stranded (ds) PCR fragments, containing the normal sequences, have been examined for their gene correction abilities by the small fragment homologous replacement (SFHR) method.2–7) Correction of mutations in the CFTR and dystrophin genes by the SFHR method has been examined and partial gene corrections were obtained.3,4,6,7) The corrected genes should be properly expressed under the control of the original promoter. When corrected, the therapeutic effects are expected to be life-long. Gain-of-function or predominant mutations could be suitable subjects for gene correction, making SFHR a highly attractive therapeutic strategy. However, the current SFHR method yielded the low correction efficiency, and thus, the correction efficiency of SFHR must be improved.

Recently, we prepared a ds DNA fragment, and single-stranded (ss) DNA fragments containing sense and antisense sequences, by restriction enzyme digestions of plasmid DNA and ss phagemid DNAs, respectively.8,9) The gene correction efficiencies of these fragments were tested with an inactivated episomal hygromycin-resistance (Hyg) and enhanced green fluorescence protein (EGFP) fusion gene containing a base substitution, as a model target (Fig. 1A). We found that the ds DNA fragment prepared by restriction enzyme digestion of plasmid DNA (dsHES, Fig. 1B) provided a 2-fold increase in the gene correction activity, as compared to the conventional PCR fragment (pcrHES). Moreover, the ss DNA fragment with the sense sequence (fSense) yielded a more than 10-fold higher gene correction frequency in comparison with pcrHES.

Frameshift mutations can inactivate genes and cause disease, and thus they are interesting targets for gene correction. In this study, we examined whether these newly designed DNA fragments were also effective in correcting frameshift mutations. Hyg-EGFP fusion genes inactivated by one base deletion and insertion mutations were chosen as model targets, and sense and antisense ss DNA fragments and ds DNA fragments were co-introduced into CHO-K1 cells with a plasmid DNA carrying the target gene (Fig. 1). The gene correction efficiency was quantitatively determined by counting the EGFP-positive and hygromycin-resistant Escherichia coli colonies, after recovery of the plasmid DNA from the transfected cells and a second transfection into bacteria. The fSense and dsHES fragments were more effective than the conventional PCR fragment (pcrHES), as in our previous results on the correction of a substitution mutation.8) The frameshift correction efficiencies of fSense were comparable to those of dsHES. These results suggest that sense ss DNA and ds DNA fragments, prepared from phagemid and plasmid DNAs, respectively, have the potential to correct frameshift mutations.

MATERIALS AND METHODS

General The pTENHES and pTENHEX plasmids, and the pBONES/Sense and pBONES/AntiSense phagemids were the same DNAs described in our previous study.5) Oligodeoxynucleotides were purchased from Sigma Genosys Japan (Ishikari, Japan) and Hokkaido System Science (Sapporo, Japan) in purified forms. VCSM13 and BL21(DE3) were from Stratagene (La Jolla, CA, U.S.A.). Sequencing reactions were conducted with a BigDye Terminator v3.1 Cycle Sequencing kit and an ABI model 377 DNA sequencer (Applied, Norwalk, CT, U.S.A.).

Plasmid Construction Phosphorylated mutagenic primers (5’-dGGCGAAGAATACGTGCTTCA and 5’-dGGCGAAGAATACGTGCTTTC, sequences corresponding to ‘codon 34’ are underlined) were annealed with the ss forms of the pALHEXP phagemid DNA,8 and the site-directed mutagenesis reactions were performed with the Altered Sites II in vitro Mutagenesis System (Promega). BamHI–Sall frag-

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mements containing an inactivated Hyg-EGFP gene were inserted into the Ncol–XhoI sites located downstream of the CMV and T7 promoters in pTriEx-3 Neo (EMD Biosciences, San Diego, CA, U.S.A.) with the aid of linker oligodeoxyri-bonucleotides (upper: 5′-dCATGGCGATCTCCTCGA, lower: 5′-dGATCTCGAGGATCG). This linker not only connects the Ncol end of pTriEx-3 Neo with the BamHI end of each fragment, but also constructs a second XhoI site (underlined sequence). The plasmid DNAs thus obtained, pTENHEdel and pTENHEins, carry the Hyg-EGFP genes with one base deletion (−C) and insertion (+G) mutations, respectively (Fig. 1A). These plasmid DNAs were purified by the Endofree Plasmid Mega kit.

Preparation of DNA Fragments for Gene Correction
The 606-bp ds fragments, pcrHES and dsHES, were prepared by PCR amplification and XhoI digestion, respectively, from pTENHES containing the normal Hyg-EGFP gene, as described previously.8) The 606-nt ss fragments, fSense and fAntiS, were prepared from ss phagemids, pBSHES/Sense and pBSHES/AntiSense, respectively, by annealing with their respective scaffold oligodeoxyri-bonucleotides followed by XhoI digestion, as described.9) These DNA fragments were purified by low-melting point agarose gel electrophoresis and gel filtration chromatography. Their UV spectra were measured to confirm their purities and to calculate their yields. The concentrations were determined by the molar absorption coefficients of DNA: 1.0 OD260 equals 50 µg/ml of ds DNA or 40 µg/ml of ss DNA. These DNA fragments were heat-denatured at 98 °C for 5 min, and were immediately chilled on ice for at least 5 min until transfection.

Cell Culture and Transfection
CHO-K1 cells were maintained in DMEM/F12 (Invitrogen, Carlsbad, CA, U.S.A.) medium supplemented with 10% fetal bovine serum (FBS) and antibiotics, in a 5% CO₂ atmosphere at 37 °C, and 1×10⁵ cells were cultured in a 10-cm dish every 3 d. One day before transfection, 3×10⁵ cells, suspended in 4 ml of culture medium, were placed in a 6-cm dish.

One of these DNA fragments (10 pmol) was mixed with 125 ng (25 fmol) of a target plasmid (pTENHEdel and pTENHEins). An appropriate amount 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 µg 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 posttransfection, the cells were harvested, and the plasmid DNA was recovered, as described previously.9)

Determination of Gene Correction Frequency
Electro-competent E. coli cells were prepared essentially according to the method described in the literature.9) The electroporation of the DNA recovered from CHO-K1 cells into electro-competent DH5α cells, and the isolation of the amplified plasmid DNA were carried out as described previously.9) A 1—2-µl aliquot of the plasmid DNA recovered from the transformed DH5α cells was electroporated into BL21(DE3), and following an incubation in 1 ml of SOC medium at 37 °C for 1 h, a 50-µl aliquot of bacterial cells was diluted into 1 ml of LB medium, containing 50 µg/ml of ampicillin and 10 µM of IPTG, and the cells were further incubated for 3 h. Dilutions (10—100-fold) of the cell suspension were seeded onto LB agar plates containing 50 µg/ml of ampicillin, 10 µM of IPTG, and 50—75 µg/ml of hygromycin (+Hyg). Dilutions (1000—10000-fold) of the cell suspension were seeded onto LB agar plates containing 50 µg/ml of ampicillin and 10 µM of IPTG (−Hyg). The plates were incubated at 37 °C, and the number of colonies on the −Hyg plates was counted at 12—24 h. For the colonies on the +Hyg plates, the number of EGFP-positive colonies was counted at 48 h with a FLA2000G image analyzer (Fuji, Tokyo, Japan). Gene correction frequencies were calculated by dividing the number of EGFP-positive colonies on the +Hyg plates by the number of colonies on the −Hyg plates. Unexpectedly, a small fraction of bacterial cells carrying an inactivated Hyg-EGFP gene appeared on the +Hyg plate. However, we could exclude these false-positive colonies from the calculation by analysis with an image analyzer. In addition, the number of colonies carrying the normal Hyg-EGFP gene on the +Hyg plate was nearly identical to that on the −Hyg plate (data not shown). Thus, the dual (Hyg-resistance and EGFP-positive) phenotypic selection ensured the precise estimation of the gene correction efficiencies. Under our conditions, we could obtain similar numbers of EGFP negative colonies on the −Hyg plates (ca. 10^5 colonies).

Genotypic Analysis
A cell-suspension (20 µl) of an EGFP-positive colony was boiled for 5 min at 95 °C, and after centrifugation, 2 µl of the supernatant were used as the template for PCR, using the primers (5′-dTAATACGACT-CACATAAGGG and 5′-dATCGCCTCGGCTCAGTCAAT) and recombinant Taq DNA polymerase to amplify the region containing the target position. The PCR products were then digested with PmaCI, which exclusively recognizes the sequence containing the corrected base pair were sequenced.

RESULTS

Experimental Design
We previously examined the correction of a substitution mutation by newly designed DNA fragments with a mutated Hyg-EGFP gene, in which codon 34 is TGA (termination codon) instead of the normal TCA (Ser) sequence.9) To prepare targets for frameshift correction, we also modified codon 34. The TCA sequence was converted to TCGA (the pTENHEins plasmid) and TA (the pTENHEdel plasmid) for one base insertion and deletion mutations, respectively (Fig. 1A). The former +G insertion results in the formation of a termination TAG codon at the 52nd position. The latter −C deletion generates the TAG codon at the 41st position. As shown in the previous paper, correction of these inactivated Hyg-EGFP genes in CHO-K1 cells led to the EGFP-positive and hygromycin-resistant phenotype of BL21(DE3) E. coli cells, in which T7 RNA polymerase can be inducibly expressed with IPTG upon transformation with the plasmid DNA recovered from the CHO-K1 cells.

As in our previous study, we prepared four types of DNA fragments for gene correction (Fig. 1B). PCR-amplification of a 606-bp region of the normal Hyg-EGFP gene in pTENHES, containing the 34th TCA codon, was performed with recombinant Taq DNA polymerase to yield pcrHES as the conventional ds DNA fragment (Fig. 1B). The other 606-bp ds DNA fragment (dSHES) was prepared by XhoI diges-
tion of pTENHES. The 606-nt ss DNA fragments were obtained by XhoI digestion of ss phagemids containing sense and antisense sequences, after annealing with scaffold oligodeoxyribonucleotides complementary to the two XhoI sites within the phagemid (fSense and fAntiS, respectively).

Plasmids pTENHEins and pTENHEdel, the gene correction targets in this study, were introduced into CHO-K1 cells together with these fragments. After 48 h, the plasmid was isolated from the transfected cells, and the recovered DNA was transformed into E. coli. Due to the high transformation efficiency, the recovered plasmid was first electroporated into the BL21(DE3) strain, in which T7 RNA polymerase can be inducibly expressed with IPTG.

The Sense Single-Stranded and Double-Stranded DNA Fragments from the Phagemid and Plasmid Corrected Frameshift Mutations More Efficiently Than the PCR Fragment As in the previous study, we introduced two kinds of ds DNA fragments, dsHES and pcrHES, and two kinds of ss DNA fragments with sense and antisense sequences (fSense and fAntiS, respectively) (Fig. 1B) into CHO-K1 cells with a target plasmid. Each heat-denatured dsDNA fragment was introduced into CHO-K1 cells, and the plasmids recovered from the cells at 48-h posttransfection were used to transform the BL21(DE3) E. coli strain. The numbers of colonies on agar plates with and without hygromycin were counted to determine the gene correction frequencies. Bars indicate S.E.M. (n = 5—7). *p < 0.05; **p < 0.01; ***p < 0.001.

We first examined the correction of the +G insertion mutation with the newly designed DNA fragments. The dsHES fragment converted to the normal sequence with an efficiency of 0.21%. The fSense fragment was slightly less efficient (0.18%) than dsHES, and these newly designed fragments showed ca. 2.5-fold improved correction frequencies as compared to the conventional PCR fragment (0.08%) (Fig. 2). The fAntiS fragment was as efficient as the pcrHES fragment.

Similar results were obtained in the experiments with the pTENHEdel plasmid containing the −C deletion mutation as the gene correction target (Fig. 2). The fSense fragment corrected the deletion most effectively (0.27%), followed by the dsHES fragment (0.19%). These values were 2.3- and 1.6-fold higher, respectively, than the correction frequency with the pcrHES fragment (0.12%). Again, the fAntiS fragment was less effective than the fSense fragment.

Sequence-Specificity of ds and ss DNA Fragment-Mediated Gene Correction The gene correction judged by the phenotypical change was confirmed by sequence analysis of the Hyg-EGFP genes in EGFP-positive and hygromycin-resistant bacterial colonies. First, PmaCI digestion was carried out to distinguish between the mutated and corrected genes. Corrected, but not uncorrected, plasmids contained a PmaCI recognition site (Fig. 1A). All of the analyzed plasmids in the EGFP-positive colonies were digested by PmaCI, indicating the successful correction to TCA at codon 34 (data not shown). Sequencing of the gene also confirmed the successful correction at the same position, and indicated the absence of nucleotide alterations in the flanking sequences (Fig. 3). Thus, the ds and ss DNA fragments could correct a mutation without inducing mutations at other sites.

DISCUSSION

As an assay system for SFHR, we used plasmid DNAs carrying an inactivated Hyg-EGFP gene, the correction target gene, which offers the convenient and quantitative detection of gene correction. Quantitative data were obtained by count-
ing the EGFP-positive and hygromycin-resistant *E. coli* colonies. This assay system was successfully employed to determine the correction efficiencies with an inactivated Hyg-EFPP gene containing a substitution mutation. As described in the previous paper, gene correction does not occur in *E. coli*, because the direct introduction of the DNA fragment and the target plasmid into bacterial cells did not result in the formation of the corrected molecule.

Using this assay system, we quantitatively evaluated the activities of newly designed DNA fragments for SFHR: (1) a ds DNA fragment prepared from plasmid DNA and (2) actual ss DNA fragments with sense and antisense sequences, in the correction of frameshift mutations. In contrast to the correction of a base substitution, the predominance of the ss sense DNA fragment (fSense) over the ds fragment derived from the plasmid (dsHES) was not observed for the corrections of frameshift mutations (Fig. 2). However, they showed higher gene correction activity, as compared with the conventional PCR fragment (pcrHES). For the correction of the +G insertion mutation, the efficiencies were in the order of dsHES (0.21%) ≫ fSense (0.18%) ≫ pcrHES (0.08%). In the case of the correction of the −C deletion mutation, the efficiencies were in the order of fSense (0.27%) ≫ dsHES (0.19%) ≫ pcrHES (0.12%). These results suggest that the sense ss DNA and ds DNA fragments, prepared from the phagemid and plasmid, respectively, have the potential to correct the frameshift mutations.

The correction frequencies of the +G and −C frameshift mutations with the four kinds of DNA fragments were lower than those of the substitution mutation. In particular, the corrections with the sense ss DNA fragment for these frameshift mutations were reduced by 6.6- to 10-fold. Gene correction is thought to involve homologous recombination and/or mismatch repair, in which the pairing process is highly important. Thus, the decreased correction frequency for the frameshift mutations may be explained by weaker recognition by the proteins that perform the gene correction. Alternatively, the corrections of substitution and frameshift mutations with the DNA fragments used, especially fSense, might follow different mechanistic pathways.

The DNA fragments prepared from phagemid and plasmid DNAs possess an additional advantage, in that they have a highly accurate nucleotide sequence as compared with DNA amplified *in vitro*. Since replication in living cells is extremely accurate, the possibility that the ss DNA fragment contained an unexpected mutation within the fragment was quite low. Indeed, no unexpected sequence alterations existed within the sequenced region (data not shown). Thus, these DNA fragments could be a safe tool in gene therapy.

In our present and previous studies, fSense showed higher gene correction efficiencies than fAntiS (Fig. 2), which contradicts the observation in the previous studies using modified ss oligonucleotides. This discrepancy might be due to differences in target site sequences, targeted genes, or length of DNA fragments. In addition, dsHES showed higher gene correction efficiencies than pcrHES (Fig. 2). In the case of fSense, N6-methyladenine within the ss DNA fragment enhanced the gene correction efficiency (Tsuchiya *et al*., unpublished results). The ds DNA fragment prepared from the plasmid DNA amplified in a *dam*− *E. coli* strain possesses a perfectly methylated sequence, while no methylation is expected for the PCR fragment, because only unmodified deoxyribonucleotides were included in the PCR. The more efficient gene correction by dsHES than by pcrHES could also be interpreted as a consequence of the differences in the methylation status.

The correction efficiency toward frameshift mutations obtained with our newly designed DNA fragments was less than 1%. Thus, further enhancement of the correction efficiency, such as increased exonuclease-resistance, which could be achieved by modifications of the fragment is necessary. Studies toward this goal are now in progress.

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**REFERENCES AND NOTES**

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