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Targeted Correction of a Defective Selectable Marker Gene in Human Epithelial Cells by Small DNA Fragments

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A novel gene targeting strategy, small fragment homologous replacement (SFHR), has been used to correct specific genomic lesions in human epithelial cells. The frequency of targeting was estimated to be 1–10%. However, given the genomic target, the cystic fibrosis transmembrane conductance regulator (CFTR) gene, it is difficult to accurately quantify targeting frequency. As an alternative to targeting CFTR, targeted correction of a mutant selectable marker or reporter gene would be more amenable to accurate and rapid quantification of gene targeting efficiency. The present study evaluates the conditions that modulate SFHR-mediated correction of a defective Zeocin antibiotic resistance (Zeo^r) gene that has been inactivated by a 4-bp insertion. The conditions include delivery systems, plasmid-to-fragment ratio, fragment length, and fragment strandedness (single- or double-stranded DNA). Targeting fragments comprise the wild-type Zeo^r gene sequence and were either 410 (Zeo1) or 458 bp (Zeo3). Expression vectors containing the corrected Zeo^r gene were isolated as episomal plasmids or were allowed to stably integrate into cultured human airway epithelial cells. Correction of the Zeo^r gene was phenotypically defined as restoration of resistance to Zeocin in either bacteria or epithelial cell clones. Extrachromosomal gene correction was assayed using polymerase chain reaction amplification, restriction enzyme digestion, DNA sequencing, and Southern blot hybridization analysis of DNA from isolated prokaryotic and eukaryotic clones. Neither random sequence alteration in the target episomal gene nor random integration of the small fragments was detected. Targeted correction efficiencies of up to 4% were attained. These studies provide insight into parameters that can be modulated for the optimization of SFHR-mediated targeting.

INTRODUCTION

Gene targeting, as a strategy for gene therapy, provides a mechanism for achieving permanent, site-specific correction of DNA lesions and results in the cell-appropriate expression of the target gene (1, 2). A gene targeting strategy allows the integrity and regulation of the gene to be maintained even when *cis*-acting regulatory elements are distant from the coding sequences (3) or are within introns (4). In addition, the nonviral gene delivery strategies used to introduce the targeting DNA into cells mitigate the immune and inflammatory side-effects triggered by viral capsid antigens and unmethylated CpG sequences present in cDNA expression plasmids (5–7).

Small fragment homologous replacement (SFHR) is a gene targeting strategy that has been successfully used to target and modify genomic DNA sequences through the introduction of small fragments of DNA homologous to specific genomic loci in human epithelial cells (8–11). SFHR was initially used to correct the ΔF508 mutation, the most common lesion associated with the cystic fibrosis transmembrane conductance regulator (CFTR) gene, in CF airway epithelial cells. The DNA fragments comprised a 491-bp wild-type (wt) CFTR sequence that would replace the three deleted nucleotides that characterize the ΔF508

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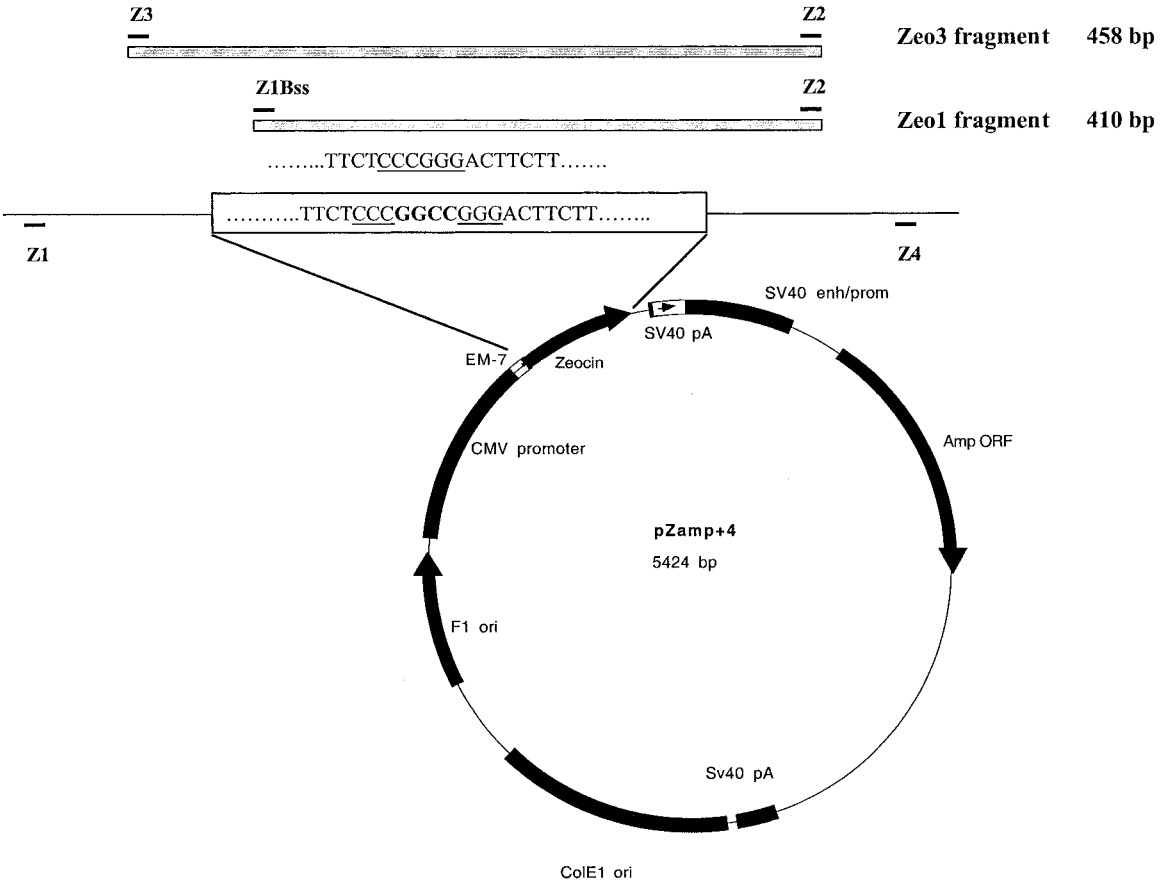

FIG. 1. Schematic representation of the defective plasmid (pZamp+4), replacement fragments, and primer locations. The defective plasmid differs from the normal plasmid (pZamp) by the addition of 4 bp in the Zeo^r coding sequence (bold letters). The Zeo1 fragment (410 bp) starts 33 bp downstream of the ATG codon of the Zeo^r gene and ends 71 bp downstream of the stop codon. The Zeo3 fragment (458 bp) starts 13 bp upstream of the ATG codon. Forward (Z1, Z1Bss, Z3) and reverse (Z2, Z4) primers are indicated.

mutation. As a result, wtCFTR mRNA expression and the functional correction of the cAMP-dependent Cl^- transport defect associated with CF were observed (8). A targeting frequency of 1–10% was indicated in this study. In a parallel study, targeting DNA fragments comprising genomic ΔF508 CFTR sequence (488 bp) were able to delete the 3 nt that constitute the ΔF508 mutation gene in the genomic DNA of normal human airway epithelial cells (10). Furthermore, a single nucleotide, ~100 bp upstream of the ΔF508 mutation, was concomitantly altered to introduce a novel restriction enzyme cleavage site for analysis.

For the successful application of SFHR as a gene therapy, optimization of the conditions for targeting is necessary. A drawback of having CFTR as the target gene is the lack of an endogenous selection mechanism that readily differentiates between targeted and parental cells and, thus, limits the ability to accurately quantify targeting frequency. As an alternative, a gene that is either critical for cell survival or makes a readily detectable protein product can be used. Transient transfection assays, employing expression vectors carrying functionally defective selectable marker or reporter genes, have been valuable for assaying targeting frequencies in other sequence-specific correction studies (12–16). Because the episomal vectors are not integrated, they can be isolated from the transfected cells and used to transform bacteria for a quantitative assessment of targeting frequency. This type of quantitative assessment is critical for the optimization of parameters that modulate SFHR efficacy.

In the present study, the frequency of SFHR-mediated correction was used as a means to evaluate those conditions that can enhance the effectiveness of SFHR in human epithelial cells. The defective Zeocin antibiotic resistance gene (Zeo^r), in a prokaryotic/eukaryotic expression vector, was cotransfected with small fragments of wild-type Zeo^r DNA into transformed CF airway epithelial cells (Fig. 1). SFHR-mediated correction was determined as restoration of Zeocin resistance after transient transfection and stable transfections in eukaryotic (human epithelial) cells. In addition, both prokaryotic and eukaryotic Zeocin-resistant clones were characterized by restriction enzyme digestion, direct DNA sequencing, and Southern blot hybridization.

MATERIALS AND METHODS

Cells. SV40-transformed (17) cystic fibrosis bronchial epithelial cells (CFBE41o-), homozygous for the ΔF508 CFTR mutation (unpublished data), were used. Cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% penicillin/streptomycin, at 37°C in 5% CO_2–95% air. All the cultures were maintained in flasks or dishes precoated with an extracellular matrix of fibronectin–Vitrogen–bovine serum albumin (18). ElectroMax *Escherichia coli* DH10B (Rec $A1^-$) cells (Life Technologies, CA) were used for the bacterial transformation studies.

Plasmid construction. The plasmid pZamp was constructed by introducing the ampicillin gene from pcDNA3 (Invitrogen, CA) (fragment *Sal*I–*Pvu*II) into the multiple cloning site of pZeoSV (*Xho*I–*Pvu*II) (Invitrogen). The pZeoSV plasmid contains the Zeocin antibiotic resistance gene under CMV (eukaryotic) and EM-7 (prokaryotic) promoters. The resultant pZamp vector was then linearized at a unique *Xma*I restriction enzyme cleavage site within the coding sequence of the Zeo^r gene. The linearized vector was incubated with Klenow enzyme to fill in the overlapping bases and then ligated. The resultant vector, pZamp+4 (Fig. 1), carries a 4-bp insertion that eliminates the *Xma*I restriction site and is no longer resistant to the Zeocin antibiotic in either prokaryotic or eukaryotic cells. The presence of the ampicillin gene allows for bacterial transformation and selection. Plasmid DNA was prepared by column chromatography (Qiagen, CA) in accordance with the manufacturer's instructions.

Fragment preparation. Two different DNA fragments, Zeo1 and Zeo3 (410 and 458 bp, respectively), were employed for targeting (Fig. 1). Both fragments contain the wt Zeo^r gene sequence and were prepared by PCR as previously described (8, 11). Primers Z1Bss (5′-GCGCGCGACGTCGCCG-GAGCG-3′) (sense) and Z2 (5′-AACAAGTTTCGAGGTCGACCCC-3′) (antisense) were used to generate the Zeo1 fragment. Primers Z3 (5′-TAG-GAGGGCCACCATGGCCA-3′) (sense) and Z2 (antisense) were used for the Zeo3 fragment. The PCR conditions were as follows: initial denaturation, 95°C/2 min; followed by 25 cycles of denaturation, 95°C/5 s; annealing, 65°C/20 s; extension, 72°C/20 s with a 7 min extension in the final cycle. The PCR mixture contained 0.2 mM dNTPs, 0.2 μM primers, 1× DMSO, 1× *Pfu* reaction buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris–HCl (pH 8.8), 2 mM $MgSO_4$, 0.1% Triton X-100, 100 ng/μl BSA), and 1 U *Pfu* DNA polymerase (Stratagene, CA). PCR products were subcloned into the pCR-Script vector (Stratagene), sequenced, and used as a template to generate preparative amounts of fragment for transfection. Production of the targeting fragment involved amplification of 1–2 ng of linearized pCRZeo1 or pCRZeo3 plasmids in a 100-μl PCR mixture containing 0.2 mM dNTPs, 0.2 μM primers, 1× DMSO, 10 mM Tris–HCl, 50 mM KCl, 2 mM $MgCl_2$, 0.01% gelatin, and 2.5 U AmpliTaq polymerase (Perkin–Elmer, CA). PCR products were purified from agarose gel, using the QIAquick II kit (Qiagen). The concentration of the PCR fragments used for targeting was determined using a Gibco BRL DNA mass ladder. DNA fragments were rendered single stranded by heat denaturation at 95°C for 10 min and, unless otherwise specified, cooled rapidly on ice before transfection.

Experimental protocol. CFBE41o- cells were cotransfected with the mutant plasmid (pZamp+4) and DNA fragments under a variety of conditions. These conditions included using DNA fragments of different sizes (Zeo1 or Zeo3), using single- or double-stranded DNA (ssDNA or dsDNA, respectively), and varying the ratios of fragment to mutant plasmid. Successful correction of the mutant sequence was assessed from both transient and stable transfections. For the transient transfections, plasmid DNA was isolated from transfected cells and used to transform bacteria. Correction frequency was determined as the ratio of Zeo^r to total Amp^r colonies. Control bacterial transformations included mixing extracts from epithelial cells transfected either with mutated plasmids or with DNA fragments alone. Assessment of stable transfections involved selection of epithelial cell clones containing corrected copies of the Zeo^r gene. Clones from both transient and stable transfections were grown and genotypically analyzed by PCR amplification, restriction enzyme cleavage, DNA sequencing, and/or Southern blot hybridization.

Transfection. Cells were transfected with the cationic lipid DOTAP (Boehringer Mannheim), SuperFect (Starburst dendrimer; Qiagen), or electroporation using different molar ratios of pZamp+4 plasmid and targeting fragments. Cells were grown either on six-well plates (DOTAP and SuperFect) or in T75 flasks (electroporation) until they reached 70–80% confluence.

Briefly, plasmid DNA–DOTAP complexes were formed in 100 μl of serum-free MEM at a negative to positive charge ratio of 1:4 (3 μg plasmid DNA:27.6 μg DOTAP). Complexes were allowed to form for 20 min and then added to the cells in serum-free medium (1 ml final volume). After 4 h, the medium was replaced with 2 ml of complete medium. Cells were grown at 37°C overnight and again transfected the following day with different amounts of the DNA fragment (heat-denatured ssDNA or undenatured dsDNA) that was complexed with DOTAP at the same charge ratio (1:4) used above. The fragment–DOTAP complex (100 μl) was then added to each well for an additional 4 h in 1 ml of serum-free medium. After washing the cells with Hepes-buffered saline (HBS), 2 ml of complete medium was added to each well. The two transfections were performed on separate days to reduce cytotoxicity. However, a 6-h period was sufficient for the cells to recover from transfection-induced stress and gave similar frequencies compared to waiting 12 h before the second transfection (data not shown). Additional experiments using plasmid–DOTAP–fragment complex yielded a correction frequency 10-fold lower than when the transfections were carried out in two steps. Plasmid DNA–SuperFect complexes were formed using a ratio of 3 μg plasmid DNA:6 μl SuperFect. Different amounts of the DNA fragment–SuperFect complex were then added as described above. Transfections were carried out according to the manufacturer's instructions when using SuperFect or DOTAP.

Electroporation transfections were carried out as described previously (8). Approximately 10^7 cells were trypsinized, washed with cold PBS (Mg^{2+} and Ca^{2+} free), and then resuspended in 800 μl of the same PBS solution. From 6 to 18 μg of plasmid DNA (6 μg) was mixed with varying amounts of DNA fragment and added to the cell suspension. The total amount of DNA was kept constant (60 μg) in each set of experiments using carrier DNA (i.e., unrelated fragment). The cell–DNA mixture was then incubated on ice for 10 min and electroporated at 4°C using a BTX 300 Gene Pulser at a setting of 500 μF, 240 mV, and 200 ms in 0.4-cm transfection cuvettes. Cells were incubated on ice for an additional 10 min, diluted 1:20 in complete MEM, and seeded into three T75 flasks.

Analysis of transient transfection. After transfection, cells were cultured at 37°C for 48–72 h and then harvested to isolate plasmid DNA. Briefly, cells were trypsinized and washed once with HBS. Episomal plasmid DNA was extracted using a modified alkaline lysis procedure (19). When specified, plasmid DNA was also extracted from isolated nuclei (20). Plasmid DNA was then incubated with RNase A at 37°C for 30 min and loaded onto a 0.7% SeaPlaque low-melting agarose gel. The relaxed and supercoiled forms of the plasmid (Fig. 2) were extracted from the agarose gel with the QIAquickII kit (Qiagen). A 10-μl aliquot of each sample was used to transform *E. coli* DH10B cells by electroporation using a Bio-Rad Gene Pulser II (settings: 25 μF, 200 ohm, and 1800 V; 0.1-cm cuvettes). After a 1-h incubation at 37°C, aliquots of the transformed bacteria cells were plated onto low-salt L-Broth (LB) (pH 7.5) plates supplemented with Zeocin (25 μg/ml) and ampicillin (100 μg/ml) or with ampicillin alone. After an overnight incubation at 37°C, colonies were counted and targeting frequency was determined. The frequency of correction was defined as the number of Zeo^r colonies divided by the total number of Amp^r colonies in each experiment. The numbers of Zeo^r colonies were normalized to correct for variations in plating efficiency. Individual bacterial clones were randomly picked and grown overnight in 5 ml LB plus antibiotics. Plasmid DNA was isolated with a Wizard miniprep purification system (Promega, WI). A 200-ng plasmid sample from each clone was digested with *Xma*I at 25°C and analyzed on a 0.8% agarose gel. In addition, 2 ng of each plasmid sample was used as template for a PCR analysis. The two primers used in the PCR analysis were outside the region of homology defined by the targeting fragment: primer Z1 (5′-TACGACTCACTATAGGAGGGCC-3′) (sense) and primer Z4 (5′-CTACTCAAACCTGTTTGGTGTTG-3′) (antisense). The PCR conditions were as follows: initial denaturation, 95°C/2 min; followed by 25 cycles of denaturation, 95°C/5 s; annealing, 60°C/20 s; extension, 72°C/20 s; with a 7-min extension in the final cycle. All PCR amplifications were carried out in a Perkin–Elmer Thermal Cycler (9600). The PCR amplification products (574 bp) were digested with *Xma*I and analyzed on a 1.8% agarose gel. In addition, an aliquot of the isolated plasmid DNA was sequenced with an ABI 373A sequencer in both directions, using primers Z1 and Z4.

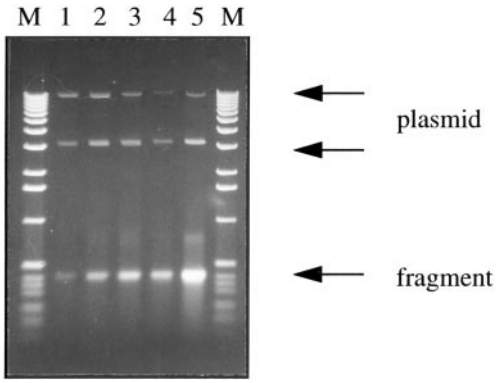

FIG. 2. Electrophoretic analysis of plasmid DNA from the total cell lysate following DOTAP or SuperFect transfection. The molecular weight marker (1-kb ladder) is in the M lanes. Lanes 1–5 represent the lysate from separate transfections with different plasmid-to-fragment ratios (1:2, 1:5, 1:10, 1:30, 1:60, respectively). The upper arrows indicate the relaxed and supercoiled forms of the plasmid DNA, respectively, and the lower arrow indicates residual targeting fragment present in the cell lysate.

Analysis of stable transfection. After transfection (48 h), the CFBE41o- cells were plated into 10-cm dishes and exposed to Zeocin selection (50 μg/ml). Selective medium was replaced with fresh Zeocin-containing medium every 2 days. Cells were grown until they formed colonies (~100 cells/colony) and isolated using cloning cylinders. Individual clones of Zeo^r epithelial cells were subcultured into T75 flasks and grown under continuous selection to 90% confluence. Genomic DNA was isolated from each epithelial cell clone and analyzed by PCR and/or Southern blot hybridization. PCR analysis entailed amplification of 200 ng of genomic DNA with Z1 and Z4 primers under the conditions described above. The amplification product was digested with *Xma*I at 25°C and analyzed on a 1.8% agarose gel. Southern blot hybridization analysis involved digesting 10 μg of DNA from individual clones with *Eco*RI and subsequent electrophoresis on a 0.8% agarose gel in TAE buffer (0.04 M Tris-acetate, 1 mM EDTA). The DNA was transferred from the gel to a nylon membrane (Hybond-N) by capillary action and crosslinked to the membrane using the UV Stratalinker 1800 (Stratagene). Hybridization was performed as previously described (21) at 65°C for 16 h, with two different radioactively randomly labeled probes. The first probe was a 613-bp CMV-specific probe generated by PCR amplification using the same conditions mentioned above with primers CM3, 5′-CATAACTTACGGTAAATGGCCCG-3′ (sense), and CMV4, 5′-CGTTCCAATGCACCGTTCCCG-3′ (antisense). The first probe was removed by washing the membrane at 100°C in 0.1% SDS for 5 min. The second probe, the 410-bp Zeo1 fragment, was specific for Zeo^r sequences.

RESULTS

Molecular Characterization of Transient Transfection

Targeting frequency was determined from the transient transfection experiments. However, the accuracy of this measurement was dependent on the purification protocol. Due to residual contaminating fragments present in all cell lysates (Fig. 2), it was necessary to gel purify extracted plasmid from the lysates before bacterial transformation. If the plasmid is not gel purified, it appears as if the bacteria can facilitate homologous replacement between the residual fragment and the defective plasmid after entry into the bacteria (data not shown). Agarose gel analysis of cell lysates also indicated that linear plasmid and genomic DNA were absent and that the extracted plasmid was either in a supercoiled or in a relaxed form (Fig. 2). Estimates based on comparison to a mass ladder indicated a plasmid recovery between 1 and 2% of the total plasmid DNA transfected.

The presence of an intact Zeo^r gene in the extracted plasmids was indicated by the cleavage of plasmid DNA with *Xma*I (Fig. 3A), thus showing restoration of the wild-type sequences in the mutant pZamp+4 plasmid. Correction was also verified through *Xma*I digestion of the PCR

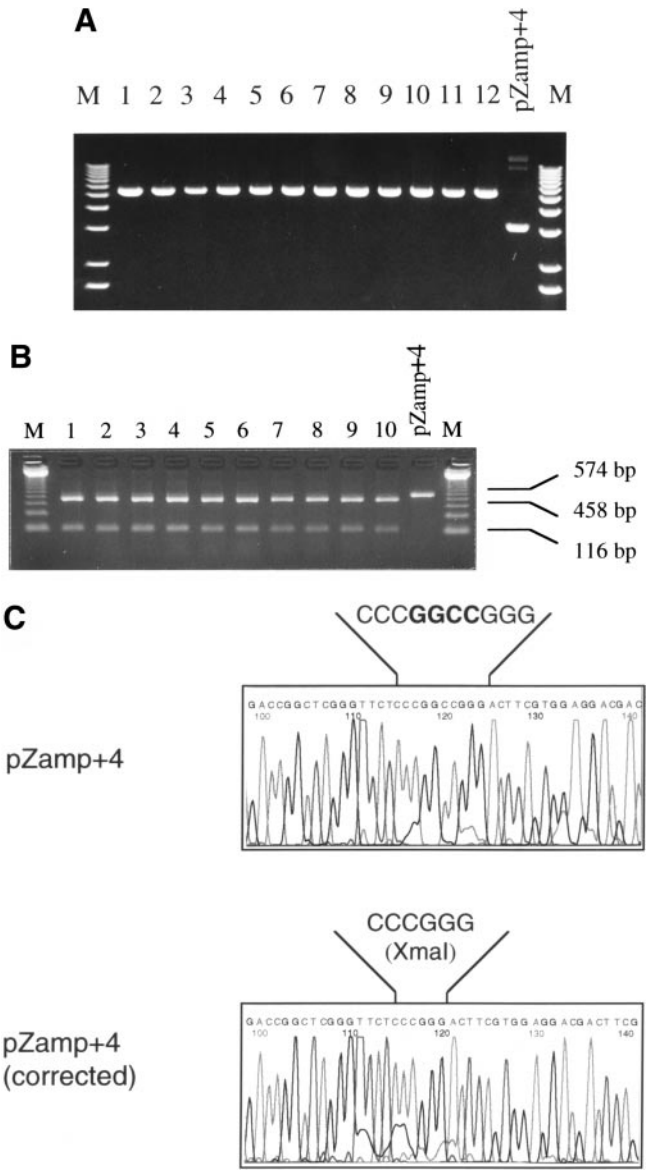

FIG. 3. Molecular analysis following transient transfection with pZamp+4 and Zeo1 fragments. (A) *Xma*I digestion analysis of plasmid DNA isolated from Zeo^r bacterial clones. Lane M, molecular weight marker (1-kb ladder); lanes 1–12, Zeo^r clones (corrected plasmid); lane 13, defective plasmid (pZamp+4) control. (B) *Xma*I digestion analysis of PCR products (primers Z1 and Z4). Lane M, molecular weight marker (123-bp ladder); lanes 1–10, Zeo^r clones (corrected plasmid); lane 11, defective plasmid (pZamp+4) control. (C) Direct sequence analysis of the defective plasmid (pZamp+4) and an SFHR-corrected Zeo^r clone. Bold letters indicate the 4-bp insertion in the pZamp+4 plasmid that subsequently disrupts the *Xma*I cleavage site.

TABLE 1

Supercoiled DNA Correction Frequency

Gene delivery system	Fragment type	Plasmid: fragment	Targeting frequency ($n = 3$)	Standard deviation
DOTAP	ssZeo1	1:5	2.37×10^{-5}	0.87×10^{-5}
		1:10	4.93×10^{-5}	0.82×10^{-5}
		1:30	2.70×10^{-5}	0.29×10^{-5}
	dsZeo1	1:5	4.93×10^{-5}	1.39×10^{-5}
		1:10	3.57×10^{-5}	1.33×10^{-5}
	None		0	0
SuperFect	ssZeo1	1:5	5.93×10^{-5}	1.59×10^{-5}
		1:10	2.33×10^{-5}	0.68×10^{-5}
		1:30	2.50×10^{-5}	1.30×10^{-5}
		1:50	2.73×10^{-5}	1.91×10^{-5}
		1:60	7.10×10^{-5}	1.42×10^{-5}
	dsZeo1	1:5	4.47×10^{-5}	1.35×10^{-5}
		1:10	4.63×10^{-5}	0.76×10^{-5}
	None		0	0
Electroporation	ssZeo1	1:10	2.75×10^{-3}	0.42×10^{-3}
		1:100	1.17×10^{-2}	0.24×10^{-2}
	dsZeo1	1:10	1.17×10^{-2}	0.09×10^{-2}
	ssZeo3	1:10	4.07×10^{-2}	0.68×10^{-2}
	None		0	0

Note. SFHR-mediated correction of supercoiled episomal pZamp+4 vector after transient transfections with replacement DNA fragments. ss, single-stranded fragment; ds, double-stranded fragment.

product after amplification of the plasmid DNA with Zeo^r-specific primers (Fig. 3B). Only plasmid isolated from Zeo^r bacterial clones showed the expected 458- and 116-bp bands. Finally, the Zeo^r gene in 10 randomly chosen Zeo^r bacterial colonies was sequenced in both directions. Removal of the 4-nt insertional mutation was observed in each case (Fig. 3C) and no additional sequence alterations were detected along the entire Zeo^r gene.

Following transfection of cells with DNA–DOTAP or DNA–SuperFect complexes, targeting frequencies ranging from 1.0×10^{-5} to 8.0×10^{-5} were observed (Table 1, rows 1–14). This frequency was independent of the time at which the plasmid was isolated, whether at 48 or at 72 h, after transfection. Neither bacterial-mediated correction nor spontaneous reversion was observed (Table 1, rows 6, 14, 19). In addition, targeting frequency appeared to be independent of the single- or double-stranded character of the fragment or of the plasmid-to-fragment molar ratio within the range tested. A dramatic 100- to 1000-fold improvement in SFHR efficiency was observed when cells were transfected by electroporation (Table 1, rows 15–19). For example, a targeting frequency of ~0.3% was indicated using the ssZeo1 fragments at a plasmid-to-fragment molar ratio of 1:10. In subsequent SFHR studies, electroporation was used to further evaluate the effects of the plasmid-to-fragment ratio, fragment length, and fragment strandedness (ss or dsDNA) on the targeting frequency. The frequency improved to ≥1% (a 5-fold improvement) with ssZeo1 fragments by increasing the plasmid-to-fragment molar ratio to 1:100. A comparable efficiency (~1%) was achieved when dsZeo1 fragments were used and the plasmid-to-fragment ratio was 1:10 (Table 1). The highest replacement frequency (4%) was observed with ssZeo3 fragments at a plasmid-to-fragment ratio of 1:10 (Table 1).

A potential issue in using artificial vehicles for delivery is that the transfected DNA may be retained in endosomes and other cytoplasmic organelles and not be efficiently transferred into the nucleus (22, 23). To test whether this was a factor, control studies with wild-type pZamp vectors were carried out. The vector was transfected into cells either via artificial vehicles or by electroporation and then extracted from the cytoplasmic compartment after 48 h. There were abundant Amp^r colonies present in the extract following transfection with artificial vehicle, whereas there were significantly fewer colonies in the extracts following electroporation. This result suggests that intact plasmid is retained in the cytoplasm and possibly protected from degradation after transfection with artificial vehicles. Thus, accurate assessment of the targeting frequency becomes compromised. The total cell lysates will contain Amp^r, defective plasmid that has not entered the nucleus and has, therefore, not been accessible to the homologous replacement machinery. To elaborate on this point, further experiments were performed to determine the extent to which the assessment of targeting frequency could be influenced by the presence of cytoplasmic plasmid. In these studies, total cell extracts were compared to nuclear extracts in terms of their relative targeting frequencies. The frequency increased 100-fold in nuclear lysate compared to the whole-cell lysate from cells transfected with artificial vehicles (Table 2). There was no difference in frequency between the nuclear and the total-cell lysates of electroporated cells.

Molecular Characterization of Stable Transfection

In parallel experiments using a plasmid-to-fragment (dsZeo1) ratio of 1:10, 20 unique Zeo^r epithelial cell

TABLE 2

Correction Frequency: Total-Cell vs Nuclear Lysates

Gene delivery system	Lysate	Fragment type	Targeting frequency ($n = 2$)	Standard deviation
DOTAP	Nuclear	ssZeo1	3.05×10^{-3}	0.95×10^{-3}
	Total		1.55×10^{-5}	0.15×10^{-5}
SuperFect	Nuclear	ssZeo1	2.35×10^{-3}	0.35×10^{-3}
	Total		2.45×10^{-5}	0.05×10^{-5}
Electroporation	Nuclear	ssZeo3	4.25×10^{-2}	0.75×10^{-2}
	Total		3.40×10^{-2}	0.20×10^{-2}

Note. SFHR-mediated correction as determined from nuclear and total-cell lysates after transient transfection with a 1:10 plasmid: fragment molar ratio.

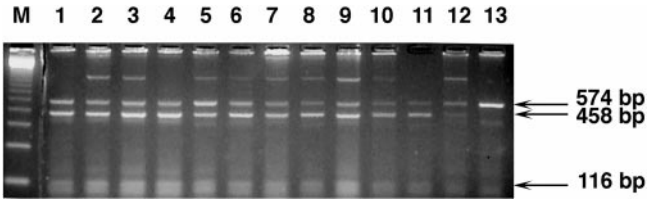

FIG. 4. PCR and restriction enzyme analysis of 12 individual Zeo^r epithelial cell clones. Total DNA was isolated from the cells and subjected to PCR amplification with primers Z1 and Z4 (574 bp) and the amplicon was then digested with *Xma*I. Defective plasmid will not be cut with *Xma*I (lane 13). Corrected plasmid will generate two bands, 458 and 116 bp. The presence of the 574-bp band in lanes 1–12 indicates that both corrected and uncorrected plasmid are present. Lane M, molecular weight marker (123-bp ladder).

clones were isolated by selection in Zeocin-containing medium. The clones were selected after transfection of 4.8×10^5 cells with SuperFect. PCR amplification and restriction enzyme digestion (*Xma*I) of genomic DNA from the Zeo^r epithelial clones indicate that these clones contain at least one SFHR-corrected copy of the Zeo^r gene giving rise to a minimum correction frequency of 4.1×10^{-5} (Fig. 4). In addition, the *Xma*I digest of the PCR amplicons suggests that both corrected and uncorrected copies of the gene-containing plasmids have integrated into the genomic DNA, because both uncut (574-bp) and cut (458- and 116-bp) products were present (Fig. 4).

Southern hybridization analysis showed several high-molecular-weight bands in individual clones and indicated the integration of the plasmids into different genomic loci (Fig. 5). The absence of bands representing the linear, supercoiled, or relaxed forms of the plasmid further supports the notion that the plasmid(s) has stably integrated. In addition, because the hybridization patterns using both CMV and Zeo1 probes were identical, it can be concluded that no random integration of the replacement fragments occurred. This pattern did not change even after the clones were propagated for >50 subcultures.

DISCUSSION

Using the Zeocin-based selection system as a model for SFHR, an optimized targeting frequency of ~4% was observed when the Zeo^r defective plasmid (pZamp+4) was cotransfected into human epithelial cells with single-stranded DNA fragments (ssZeo3) of wild-type Zeo^r (458 bp) at a ratio of 1:10. The frequency ranged from 0.001 to 4% and was recorded using different SFHR conditions (plasmid-to-fragment ratio, fragment length, and fragment strandedness (ss- or dsDNA)). These findings are consistent with previous studies evaluating SFHR-mediated targeting of genomic CFTR sequences that indicated a targeting frequency between 1 and 10% (8). The targeting frequencies observed in the present study are also comparable to those of studies evaluating site-specific modification of extrachromosomal target genes by small DNA fragments tethered to a triplex-forming oligonucleotide (16) and RNA/DNA chimeric oligonucleotides (13, 15). In addition, because the maximum correction frequency measured is four orders of magnitude greater than that associated with classical homologous recombination in human cells (0.0001%) (1), there is a suggestion that the mechanisms underlying SFHR are, to some degree, independent of and distinct from those involved in classical homologous recombination.

The studies presented here demonstrate the sensitivity of the assay system and the SFHR conditions that lead to effective correction. A primary factor influencing the efficiency of SFHR-mediated correction appears to be the DNA delivery system. In the present study, electroporation was 1000-fold more effective for extrachromosomal gene targeting compared to transfection with cationic liposome or dendrimer complexes. These results may, in part, be explained by the observation that uncorrected plasmids were still present in the cytoplasm at least 48 h after transfection using artificial vehicles. This plasmid DNA does not enter the nucleus to undergo SFHR-mediated exchange and would likely be protected by the delivery vehicle from exonuclease degradation. The defective, "cytoplasmic" plasmid component of the whole-cell lysate increases the number of Amp^r colonies and thus decreases the actual correction frequency. The 100-fold increase in SFHR frequency observed when bacteria were transformed with plasmid isolated from nuclei alone reinforces this notion and indicates that if plasmid enters the nucleus, the probability of SFHR is increased even when artificial vehicles are used. This artifactual increase in the number of Amp^r colonies was not a factor in the electroporation experiments. Following electroporation, there was no significant difference in targeting frequency using plasmids isolated from the nuclear or the whole-cell lysates. In addition, very few functional plasmids were detected in the cytoplasmic fraction 48 h after electroporation. This is not unexpected, because electroporated DNA is uncoated and is thus more susceptible to degradation by cytosolic nucleases. Recent studies in COS and HeLa cells support this observation and showed a 50- to 90-min half-life of plasmid DNA when it is directly microinjected to the cytoplasm (24).

The finding, in the electroporation experiments, that dsDNA fragments (dsZeo1) were 10-fold more efficient than the corresponding ssDNA fragments (ssZeo1) in facilitating homologous replacement when the plasmid-to-fragment ratio was 1:10 suggests that different enzymatic pathways mediate SFHR. This finding differs from previ-

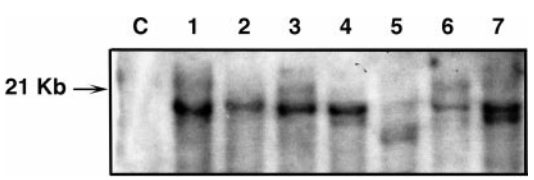

FIG. 5. Southern blot hybridization analysis of seven individual Zeo^r epithelial cell clones. All the samples were digested with *Eco*RI, which is not present in either the pZamp or the pZamp+4 plasmid. Blots were hybridized with a Zeo^r-specific probe. Lanes 1–7, Zeo^r epithelial cell clones. Lane C, CFBE control DNA.

ous studies directed at modifying genomic targets. In these studies either 165-bp DNA fragments or linearized M13 vectors were used, and dsDNA and ssDNA were equally effective in mediating targeted exchange at the genomic hypoxanthine-phosphoribosyltransferase and adenine phosphoribosyltransferase loci, respectively (25, 26). These studies used DNA-delivery vehicle complexes and showed correction efficiencies of $\sim 10^{-5}$ to 10^{-6}. Furthermore, this discrepancy could, in part, be due to nonconservative single-stranded annealing that is distinct from the mechanism purported to underlie classical genomic homologous recombination (27).

While the fragment-to-plasmid ratio appeared to have no dramatic effect in modifying the targeting frequency in cells transfected using artificial delivery vehicles, the studies using electroporation showed a 5-fold increase in targeting when the fragment-to-plasmid ratio was increased by 10-fold (ssZeo1 fragment). The results from the whole-cell extract comparison clearly show that transfection with artificial vehicles is complicated by retention and protection of the DNA complex within the cytoplasm. For this reason, electroporation should be used as a standard delivery protocol to obtain measurement of how fragment to plasmid ratio influences targeting efficiency.

A consideration in using the transient transfection expression systems to obtain an accurate quantification of SFHR-mediated correction is the cDNA character of the reporter/selection marker genes used as targets. Because these assay systems are composed of cDNA, no intron sequences are present in the replacement fragments. This could result in an underassessment of the targeting efficiency, because SFHR-mediated correction could introduce inactivating mutations into the coding region when the ends of the targeting fragments are within exons (28, 29). Fortunately, the small size (374 bp) of the selection gene chosen in our system has facilitated some degree of elucidation of this possibility. Of the two different replacement fragments, Zeo1 and Zeo3, only Zeo3 has both 5′ and 3′ ends outside of the coding region of the Zeo^r gene. It is, thus, possible that having the ends of the fragment outside the coding region, and the placement of the modifying sequences in a more central position within the replacement fragment, results in an increase the targeting frequency from 0.3 to 4%. This frequency is comparable to values obtained in correction of genomic CFTR DNA (8) and implies that SFHR-mediated modification of genomic DNA and extrachromosomal DNA may engage elements that are similar.

Finally, the selective nature of this assay system allowed for the isolation of individual Zeo^r epithelial cell clones. Southern hybridization analysis of these isogenic clonal cell populations showed a heritable integration of the plasmids for >50 subcultures. More importantly, this analysis revealed no random integration of the correcting fragments after SFHR, addressing a very relevant concern about the safety of SFHR-mediated gene targeting.

In summary, the Zeocin system has provided an effective means to accurately quantify and characterize SFHR-mediated correction. The findings of the present study support our previous results (8, 10) and have provided insight into the conditions that modulate SFHR and the pathways that underlie SFHR-mediated targeting. Varying the conditions relevant to SFHR, such as the delivery systems, and/or the length and character of the DNA fragments has been shown to influence gene targeting and can be further optimized to improve the efficiency of SFHR. SFHR appears sequence independent and can simultaneously add or delete multiple nucleotides whether or not they are adjacent. Thus, SFHR can complement other gene targeting strategies that are limited either to specific sequences or to the number of bases that can be simultaneously modified (1, 2).

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