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In vivo gene repair of point and frameshift mutations directed by chimeric RNA/DNA oligonucleotides and modified single-stranded oligonucleotides

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ABSTRACT

Synthetic oligonucleotides have been used to direct base exchange and gene repair in a variety of organisms. Among the most promising vectors is chimeric oligonucleotide (CO), a double-stranded, RNA–DNA hybrid molecule folded into a double hairpin conformation: by using the cell's DNA repair machinery, the CO directs nucleotide exchange as episomal and chromosomal DNA. Systematic dissection of the CO revealed that the region of contiguous DNA bases was the active component in the repair process, especially when the single-stranded ends were protected against nuclease attack. Here, the utility of this vector is expanded into *Saccharomyces cerevisiae*. An episome containing a mutated fusion gene encoding hygromycin resistance and eGFP expression was used as the target for repair. Substitution, deletion and insertion mutations were corrected with different frequencies by the same modified single-stranded vector as judged by growth in the presence of hygromycin and eGFP expression. A substitution mutation was repaired the most efficiently followed by insertion and finally deletion mutants. A strand bias for gene repair was also observed; vectors designed to direct the repair of nucleotide on the non-transcribed (non-template) strand displayed a 5–10-fold higher level of activity. Expanding the length of the oligo-vector from 25 to 100 nucleotides increases targeting frequency up to a maximal level and then it decreases. These results, obtained in a genetically tractable organism, contribute to the elucidation of the mechanism of targeted gene repair.

INTRODUCTION

Nucleotide exchange directed by synthetic oligonucleotides is a promising approach to functional genomics and gene therapy. Recently, the chimeric RNA/DNA oligonucleotides (COs), a molecule consisting of RNA and DNA bases configured into a double-stranded, double hairpin conformation (1,2), has been used successfully to accomplish single-base exchange. These vectors have been used to repair point and frameshift mutations in mammalian cell culture (3–9), cell-free extracts (10–12) and animal models (13–15). In addition, gene repair using COs has been carried out successfully in plants (16–18) and plant cell-free extracts (19).

Recently, we undertook a systematic approach to define the mechanism by which targeted genes are repaired. Gamper *et al.* (11) showed that the overall length of the CO, particularly in the region of complementarity, was an important factor in successful repair. Furthermore, the DNA strand of the CO mediated the initial correction event and the RNA component provided increased the stability of the reaction intermediate, a characteristic that was subsequently confirmed using *in vitro* recombination assays (20). These results prompted a more careful examination of individual sections of the intact CO. The all DNA section was found to direct gene repair as an isolated single molecule when a certain number of phosphodiester bonds connecting the terminal residues were modified. Specifically, single-stranded oligonucleotides containing three phosphorothioate bonds at the 3′ and 5′ termini were at least three times more effective than the CO in gene repair assays conducted in cell-free extracts (12). The simplicity of design and ease of purification of these new vectors have enabled more consistent results in gene repair reactions. It should be stated that while the mechanism of single-stranded directed repair is being elucidated it may be premature to link the mechanism of CO-directed repair to it.

Early attempts to develop synthetic single DNA strands as vectors focused, in a large part, on triplex-forming oligonucleotides (TFOs), which can alter specific targets in episomal or chromosomal DNA. TFOs rely on specific hybridization to uninterrupted homopurine tracts, an obvious limitation for target selection. Recently, however, bi-functional oligonucleotides that included a segment of DNA, which directed correction of a specific base, have been developed (21). In this case, the TFO contains a binding domain, which interacts stably with a purine-rich DNA sequence, tethered to the correction segment. Culver *et al.* (22) utilized this strategy for correcting the ADA mutation in human lymphocytes wherein DNA repair of the mutation was facilitated by the nucleotide excision repair (NER) pathway. As described above, Gamper *et al.* (12) have taken a similar approach, in

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which single-stranded DNA molecules with a specific number of linkage modifications were used to correct a mutation in plasmid DNA. These modifications apparently eliminate the need for homopurine runs to achieve stable binding, thereby expanding the range of viable target sequences. Such a powerful approach, when applied to the creation of individual SNPs, will be quite useful for functional genomics applications. Recently, Rice *et al.* (2,23) demonstrated targeted-gene repair in *Saccharomyces cerevisiae* using COs. Cell-free extracts from various yeast strains helped identify genes that regulate the repair process and *in vivo* experiments generally confirmed the *in vitro* results.

Yeast had been used previously as a model organism for analyzing oligonucleotide-directed base changes in plasmids (24,25). In these pioneering studies, Sherman and colleagues utilized a mutation in the *cyc1* gene as a target for repair directed by transfected (unmodified) DNA oligonucleotides. Considering the wealth of genetic mutants in *S.cerevisiae*, the availability of selectable markers and early reports of success (24,25), this system was chosen as prototype for elucidating the mechanism of targeted gene repair in the present study. As such, a new construct, containing a mutated gene, which confers resistance to hygromycin fused to a reporter eGFP gene, was built and used as the genetic target for repair in yeast. Successful correction with modified single-stranded oligonucleotide vectors (12), therefore, results in phenotypic changes in both growth properties (resistance to hygromycin) and green fluorescence. In this paper, we extend our earlier *in vitro*, biochemical studies and report the correction of point and frameshift mutations by a uniquely designed modified single-stranded oligonucleotide. Gene repair was measured by growth in selectable media, green fluorescence and by DNA sequence analysis. Parameters such as strand (sense, antisense) bias and the length of the corrective oligonucleotide have been examined and found to be critical in elevating the frequency of gene repair in *S.cerevisiae*.

MATERIALS AND METHODS

Plasmids

pAURHyg(rep)eGFP, pAURHyg(ins)eGFP and pAURHyg(del)eGFP plasmids were constructed by inserting a synthetic expression cassette containing a hygromycin gene and a fused eGFP gene into the pAUR123 shuttle vector (Panvera, WI). First, a set of three mammalian expression vectors, pHyg(rep)eGFP, pHyg(del)eGFP and pHyg(ins)eGFP, which contain a point mutation at nucleotide 137 of the hygromycin B coding sequence, were constructed. (rep) indicates a t137 to g replacement, (del) represents a deletion of nucleotide 137, and (ins) represents an insertion between nucleotides 136 and 137. All point mutations create a nonsense termination codon at residue 46. pHygeGFP plasmid (Invitrogen, CA) DNA was used as a template to introduce the mutations into the hygromycin eGFP fusion gene by a two-step site-directed mutagenesis PCR protocol. First, overlapping 5′ and 3′ amplicons surrounding the mutation site were generated by PCR for each of the point mutation sites. A 215 bp 5′ amplicon for the (rep), (del) or (ins) was generated by polymerization from oligonucleotide primer: HygeGFPf (aatacgactcactatagg), primer: Hygrepr (gacctatccacgccctcc), Hygdelr (gactatccacgccctcc) or Hyinsr (gacattatccacgccctcc), respectively. A 300 bp 3′ amplicon for the (rep), (ins) or (del) was generated by polymerization from oligonucleotide primers Hygrepf (ctgggataggtcctgcgg), Hyginsf (cgtggataatgtcctgcgg), Hygdelf (cgtggatagtcctgcgg) and HygeGFPr (aaatcacgccatgtagtg). Twenty nanograms of each of the resultant 5′ and 3′ overlapping amplicon mutation sets was mixed and used as a template to amplify a 523 bp fragment of the hygromycin gene spanning the *Kpn*I and *Rsr*II restriction endonuclease sites. The Expand PCR system (Roche, Inc.) was used to generate all amplicons with 25 cycles of denaturing at 94°C for 10 s, annealing at 55°C for 20 s and elongation at 68°C for 1 min. An aliquot of 10 µg of vector pHygeGFP, as well as 5 µg of the resultant fragments for each mutation, was digested with *Kpn*I and *Rsr*II (New England Biolabs) double digest and gel-purified in preparation for enzymatic ligation. Each mutated insert was ligated into pHygeGFP vector at 3:1 molar ratio using T4 DNA ligase (Roche, Inc.). Clones were screened by restriction digest, confirmed by Sanger dideoxy chain termination sequencing and purified using a Qiagen maxi-prep kit.

Then, a set of three yeast expression constructs, pAURHyg(rep)eGFP, pAURHyg(ins)eGFP and pAURHyg(del)eGFP, which contain the same point mutation at nucleotide 137 of the hygromycin B coding sequence as pHyg(rep)eGFP, pHyg(del)eGFP and pHyg(ins)eGFP, were created. This set was constructed by excising the respective expression cassettes by restriction digest from pHyg(x)eGFP and ligated into pAUR123 plasmid. An aliquot of 10 µg pAUR123 vector DNA, as well as 10 µg of each pHyg(x)eGFP construct, was digested with *Kpn*I and *Sal*I (New England Biolabs). Each of the DNA fragments was gel purified and prepared for enzymatic ligation. Each mutated insert was ligated into a pAUR123 vector at a 3:1 molar ratio using T4 DNA ligase, and clones were analyzed by restriction digestion and DNA sequencing as described above.

Oligonucleotides

HygGG/rev, Hyg3S/25T, Hyg3S/74T, and Hyg3S/25NT, Hyg3S/40NT, Hyg3S/74NT and Hyg3S/100NT (see Figs 1 and 3B) were targeting vectors used in this paper. HygGG/rev is a chimeric RNA/DNA oligonucleotide, the other vectors are the single-strand oligonucleotides containing three phosphorothioated bonds at the 3′ and 5′ termini and with the length of 25, 40 or 100 nt, respectively. HygGG/rev, Hyg3S/25T and Hyg3S/74T were designed to target the transcribed strand of hygromycin gene and Hyg3S/25NT, Hyg3S/40NT and Hyg3S/100NT target the non-transcribed strand. Kan3S/70T is a non-specific oligonucleotide having no complementarity to the hygromycin target gene.

Electroporation of *Saccharomyces cerevisiae*

Five micrograms of plasmid DNA was transfected into *S.cerevisiae LSY678* matα (*LSY678*, gift from Dr L. Symmington, Columbia University) by electroporation. Briefly, cells were grown at 30°C to a density of ~2×10^7 cells/ml in YPD medium, washed with 25 ml dH_2O twice and 1 ml of 1 M sorbitol once; finally, the cells were resuspended in 120 µl 1 M sorbitol. Aliquots of 40 µl yeast suspension (1×10^7 cells) were incubated with 5 µg of plasmids in ice for 5 min, then transferred to 0.2 cm cuvette and electroporated using a Bio-Rad Gene Pulser apparatus (Richmond, CA) with 1.5 kV, 25 µF,

200 Ω, 1 pulse, 5 s/pulse length. Cells were immediately resuspended in 1 ml of YPD supplemented with 1 M sorbitol and shaken at 30°C with speed of 300 r.p.m. for 6 h. 200 µl of yeast cells were spread on YPD-aureobasidin A (0.5 µg/ml) selective plates and cultured at 30°C for 3 days.

Colony PCR and *Ava*II restriction enzyme digestion

Yeast cells containing pAURHyg(rep)eGFP, pAURHyg(ins)eGFP and pAURHyg(del)eGFP plasmids were confirmed by colony PCR. Briefly, single colonies were picked and rinsed in 50 µl of PCR reaction mixture [1× PCR amplification buffer, 300 µM dNTP, 0.2 pmol primer pAUR123F (5′-tctgcacaatatttcaagc) and primer pHyg1560R (5′-aaatcagccatgtagtg), and 3.5 U *Taq* polymerase]. Samples were preheated at 95°C for 2 min, then 28 cycles of 95°C × 1 min, 55°C × 1 min, 68°C × 2 min, and a final elongation at 68°C for 6 min, cool down at 4°C. PCR products were loaded in 1.5% agarose gel to check the band of 520 bp. For these yeast cells containing pAURHyg(rep)eGFP plasmid, *Ava*II restriction enzyme digestion was performed for double check. An aliquot of 15 µl of colony PCR product was digested with *Ava*II at 37°C for 2 h, then loaded in 1.5% agarose gel to visualize the bands (270, 210 and 40 bp) after digestion.

Episomal targeting of mutated hygromycin

Five micrograms or 220 pmol of oligonucleotides was electroporated into *S.cerevisiae* (*LSY678*) containing pAURHyg(rep)eGFP, pAURHyg(ins)eGFP or pAURHyg(del)eGFP plasmids by the same protocol as previously mentioned, except that, for each sample, 200 µl of yeast cells were spread on YPD-hygromycin (300 µg/ml) plates and another 200 µl of 10^5 diluted yeast cells on YPD-aureobasidin A (0.5 µg/ml) plates at the same time, then cultured at 30°C for 3 days. Colony counts are determined using an AccuCount™ 1000 (Biologics, Inc.) and tabulated from three or four independent experiments, done in triplicate. Variation in colony numbers was never more than ±15%.

Corrected plasmid rescue and sequencing

Rescue of hygromycin resistance at nucleotide 137 was confirmed by DNA sequencing. Colonies were picked from a hygromycin plate and grown in 5 ml YPD at 30°C overnight. Cells were collected by centrifugation, 0.2 ml of Buffer A (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA pH 8.0), 0.2 ml phenol:chloroform (25:24) and 0.2 ml of glass beads (Sigma) were added then vortexed for 2 min, spun down, ethanol precipitated and redissolved in dH_2O. Resuspended plasmid (5 µl) was transformed to 20 µl competent bacteria (*Escherichia coli* DH10B) by a Cell Porator apparatus (Life Technologies) as described by the manufacturer. Each mixture was transferred to a 1 ml SOC culture and incubated at 37°C for 1 h, and a 200 µl of sample was spread on ampicillin (100 mg/ml) plate and cultured at 37°C overnight. Resultant colonies were picked and grown in 5 ml LB broth at 37°C overnight, plasmids were isolated by Concert™ rapid plasmid mini-prep kit (Life Technologies), 5 µl of mini-prep product was used as a template for Sanger dideoxy sequencing using an ABI prism kit as specified by the manufacturer on an automated ABI 310 capillary sequencer.

Observation of green fluorescence protein expression

Colonies were picked from hygromycin plates, cultured in 5 ml YPD medium for 1–3 h, then 50 µl of yeast cell suspension was inoculated into 950 µl 1 M sorbitol and loaded into an 8-chamber slide (Nunc), incubated at 30°C for 30 min. Green fluorescence indicating the presence of fusion protein was visualized with Zeiss LSM 510 confocal microscope at a band-pass range of 505–550 nm.

RESULTS

Gene repair in *S.cerevisiae* was measured by using an episomal target, plasmid pAURHyg(rep)eGFP, which contains a point mutation in the gene conferring resistance to hygromycin. This plasmid, depicted in Figure 1, contains origins of replication for growth in bacteria and yeast as well as a CEN4 element to maintain low copy number (12). The target is a fusion gene created by joining an eGFP gene and hygromycin resistance gene. Upon correction, the expression of the fusion gene exhibits resistance to hygromycin selection and green fluorescence. Also shown in Figure 1 is the relevant sequence of the wild-type TA*T* (Y) codon which has been altered to a TA*G* (*), stop codon. HygGG/rev (11,23) is a CO designed to repair the stop codon, reversing hygromycin sensitivity. Hyg3S/25T, Hyg3S/74T and Hyg3S/74NT are also depicted. These single-stranded DNA molecules are 25, 40, 60, 74 or 100 bases in length and contain three phosphorothioate linkages at each termini; they are designed to convert TA*G*→TA*C* thereby restoring function (resistance to hygromycin and expression of eGFP). Hyg3S/74T and Hyg3S/74NT differ only in that one (Hyg3S/74T) is complementary to the transcribed strand while the other is targeted to the non-transcribed strand. Kan3S/70T is a single-stranded oligonucleotide, bearing no complementarity to the target sequence in pAURHyg(rep)eGFP.

Repair of a point (replacement) mutation

pAURHyg(rep)eGFP was introduced into *S.cerevisiae* (strain *LSY678-Δmat*α) (2,23) by electroporation and maintained stably at low copy number under aureobasidin selection. As a control, pAURHyg(+)eGFP, a plasmid containing the wild-type version of the fusion gene, was also introduced as a stable episome into a separate *LSY678* strain. For each experiment, an equal volume of cells is dispensed onto agar plates containing aureobasidin because the gene encoding resistance to aureobasidin is present in pAURHyg(rep)eGFP. The number of aureobasidin-resistant clones serves to normalize the number of cells per reaction surviving electroporation. Figure 2 displays hygromycin selection of cells containing the pAURHyg(rep)eGFP or pAURHyg(+)eGFP. Yeast, containing the mutant plasmid spread on plates laden with hygromycin, did not survive. Introduction of the wild-type plasmid into the same strain resulted in heavy growth (TNTC). These data confirm that the system is selectable and that colony growth is dependent on the presence of functional expression of the hygromycin fusion gene.

Since the gene repair activity outlined in this manuscript relies on oligonucleotide action in yeast, cellular uptake of these molecules was measured. Oligonucleotides were synthesized containing a fluorescent (Texas-Red) tag on the 5′ end. These labeled vectors were electroporated into *LSY678* cells as

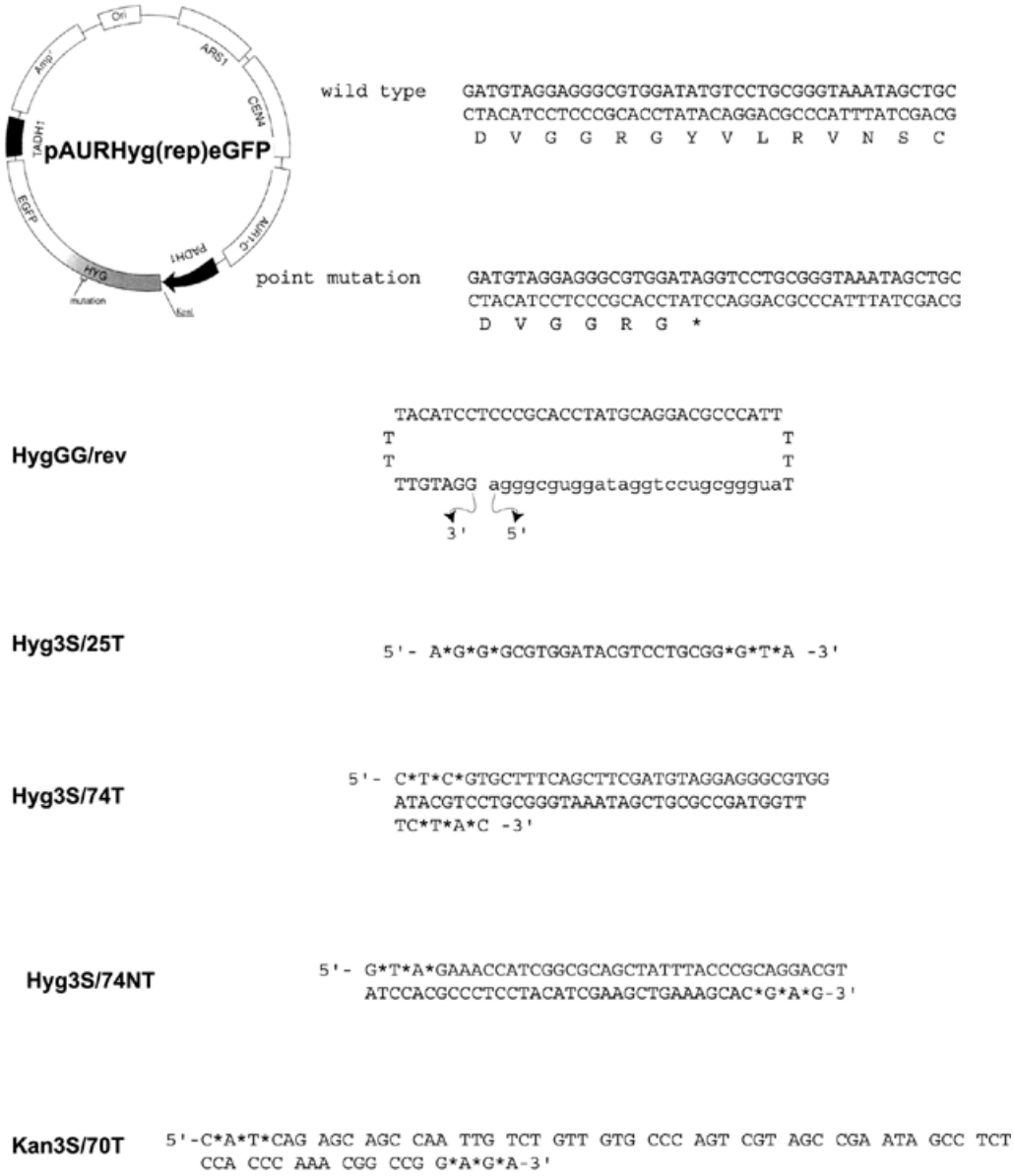


Figure 1. Model systems for gene repair utilize plasmids pAURHyg(x)eGFP and oligonucleotides. Plasmid pAURHyg(x)eGFP has a synthetic expression cassette which contains a hygromycin B gene and an eGFP gene fused to the alcohol dehydrogenase (ADH1) constitutive promoter and a selective marker of Aur1-c. Plasmid pAURHyg(rep)eGFP contains a point mutation at nucleotide 137 of hygromycin B coding sequence, which make a replacement mutation of TA(*T*) (Tyrosine) to TA(*G*) (stop codon, symbolized as '*' in frame). Correction of pAURHyg(rep)eGFP requires the replacement of the mutant G residue with a C residue. HygGG/rev is an RNA/DNA chimera with its DNA part targeting the transcribed strand of hygromycin gene; lower case letters indicate RNA bases whereas upper case ones indicate DNA residues. Hyg3S/25T, Hyg3S/74T and Hyg3S/74NT are single-stranded oligonucleotides with the length of 25 and 74 nt, respectively. T, oligonucleotides targeting the transcribed strand of hygromycin gene; NT, an oligonucleotide designed to target the non-transcribed strand. Kan3S/70T is a non-specific molecule with the length of 70 nt. *, phosphorothioate linkages within the oligonucleotide, located at both 3′ and 5′ termini.

described in Materials and Methods and visualized by confocal microscopy. Figure 2B illustrates electroporated yeast cells containing the fluorescent oligonucleotides. The majority of cells in each field of vision appear orange–red indicating the presence of the vector. Since the pictures are imaged on a Zeiss 63 X C-Apochromate water immersion lens, layers of cells are evident and may appear colorless since they are out of the focused plane. We estimate that >85% of *LSY678* yeast cells import the oligonucleotide.

The yeast strain containing the mutated plasmid was treated with various targeting vectors by electroporation using conditions previously optimized (23) and grown under hygromycin selection. When Hyg3S/25T was electroporated into pAURHyg(rep)eGFP, the mutation was corrected enabling resistant colonies to grow. Similar, albeit lower, colony counts were observed when the chimera, HygGG/rev, was used to direct correction. The number of colonies arising, however, when Hyg3S/74T was electroporated, was >90-fold higher

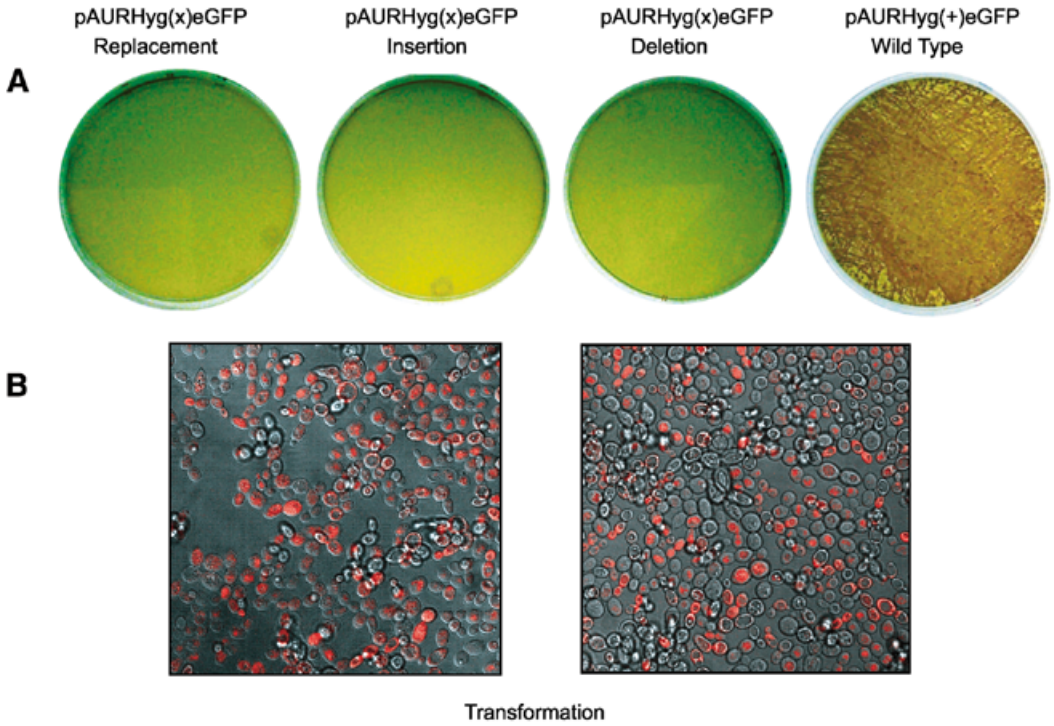


Figure 2. *In vivo* targeting hygromycin selection of *S.cerevisiae*. Five micrograms of pAURHyg(rep)eGFP, pAURHyg(ins)eGFP or pAURHyg(del)eGFP was transformed into *LSY678* matα yeast cells by electroporation, spread on hygromycin selective-YPD agar plates and cultured at 30°C for 3 days as described in Materials and Methods. No background was observed on plates in which *LSY678* matα yeast cells bearing pAURHyg(rep)eGFP, pAURHyg(ins)eGFP and pAURHyg(del)eGFP were spread, whereas *LSY678* matα yeast cells harboring pAURHyg(wt)eGFP grew confluently. (**B**) Five micrograms of Texas-Red labeled vectors (fluorescent) was electroporated into yeast cells. Samples were loaded into a Lab-Tek II chambered coverglass system and imaged with a Zeiss 63 X C-Apochromate water immersion lens. Cells were imaged on a Zeiss inverted 100 M Axioskop equipped with Zeiss 51D LSM confocal microscope and a krypton–argon laser (488 and 568 nm excitation lines) by using the 568 nm excitation line with a 590 nm longpass emission filter for fluorescence detection.

than the chimera and out-performed Hyg3S/25T by 4–5-fold (Table 1). Since 10^5 cells were electroporated, this reflects the targeting efficiency of Hyg3S/74T approximates 0.016%. This frequency may actually be higher since we are assuming every yeast cell is competent to take up the oligonucleotide and catalyze gene repair. The non-specific vector Kan3S/70T did not catalyze gene repair at any detectable level.

Since vector Hyg3S/74NT was the most active in conversion, we tested the effect of varying its concentration in the reaction. The frequency of correction was dose dependent; increasing the level of transfected oligonucleotide initially produced more hygromycin-resistant colonies (Fig. 3A). But, at the highest level (10 µg), the number of colonies exhibiting hygromycin resistance decreased. This result is due, in all likelihood, to the reduction in yeast cell survival when exposed to high amounts of oligonucleotide (data not shown). Hence, a window of vector concentration must first be established in order to obtain optimal targeting efficiency.

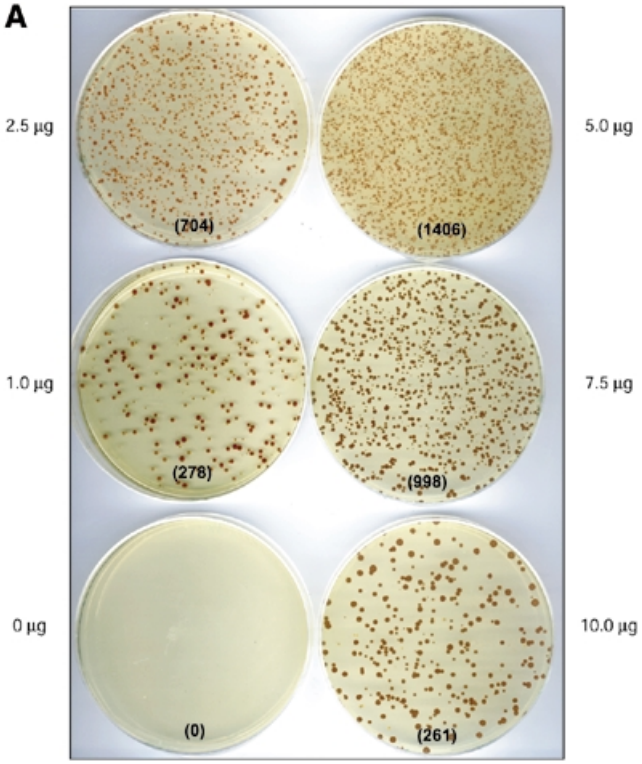
Previous results demonstrated that increasing the length of chimeric oligonucleotides elevated the level of directed gene repair (11). Hence, we tested this parameter of vector structure in the current system by varying the length of Hyg3S/74NT (see Fig. 1). Vectors composed of 25, 40, 60, 74 and 100 nt, respectively, were examined for gene repair of pAur Hyg(rep) eGFP in *LSY678*. As shown in Figure 3B, a steady increase in hygromycin-resistant colony members is observed as a function of extending the length of Hyg3S/74NT until a slight decrease is seen with the vector of 100 nt in length. Since equimolar amounts of vector were used in each reaction, this decrease is not due to toxic effects on the cell. Rather, the

Table 1. Gene repair of a point mutation in *S.cerevisiae*

Yeast strain	Plasmid	Vector (5 µg)	Average colony count/10^{-5}	
LSY678	pAURHyg(rep)eGFP	–	0	(0)
LSY678	pAURHyg(rep) eGFP	Kan3S/70T	0	(0)
LSY678	pAURHyg(rep) eGFP	HygGG/rev	3	(0.02)
LSY678	pAURHyg(rep) eGFP	Hyg3S/25T	64	(0.44)
LSY678	pAURHyg(rep) eGFP	Hyg3S/74T	280	(1.61)

Strain LSY678 (10^{-5} cells) containing plasmid pAURHyg(rep)eGFP was electroporated with 5 µg of the indicated vector. Average colony counts from four experimental plates are presented. The numbers in parenthesis indicate a correction frequency value which is obtained by dividing the number of hygromycin-resistant colonies by the number of aureobasidin-resistant colonies (per 10^{-5}) obtained per reaction. These values represent an average of four experiments with a variation of ±10%.

A



B

Hyg3S/25NT

5'-T*A*C*CCGCAGGACGTATCCACGC*C*C*T-3'

Hyg3S/40NT

5'-A*G*C*TATTTACCCGCAGGACGTATCCACGCCCTCCTAC*A*T*C-3'

Hyg3S/74NT

5'-C*T*C*GTGCTTTCAGCTTCGATGTAGGAGGGCGTGGATACGTCCTGCGGGTAAATAGCTGCGCCG
ATGGTTTC*T*A*C-3'

Hyg3S/100NT

5'-C*A*T*AACGATCTTTGTAGAAACCATCGGCGCAGCTATTTACCCGCAGGACGTATCCACGCCCTC
CTACATCGAAGCTGAAAGCACGAGATTCTTCGCC*C*T*C-3'

The effect of vector length on the repair of pAURHyg(rep)eGFP

Strain	Amount	Vector	Average Colony Count ($x10^{-5}$)
LSY678*	0	-	0
LSY678*	220pmoles	Hyg3S/25NT	66 (0.39)
LSY678*	220pmoles	Hyg3S/40NT	177 (1.05)
LSY678*	220pmoles	Hyg3S/74NT	539 (3.20)
LSY678*	220pmoles	Hyg3S/100NT	305 (1.82)

The indicated Hyg3S vectors were electroporated into LSY678* containing pAURHyg(rep)eGFP and the number of hygromycin-resistant colonies determined directly and as a function of aureobasidin-resistant colonies ($x10^{-5}$) in parenthesis.

Table 2. Repair of pAURHyg(rep)eGFP by Hyg3S/74T or Hyg3S/74NT

Strain	Amount of oligs (μg)	Hyg3S/74T	Hyg3S/74NT	Fold
LSY678	0	0	0	0
LSY678	1.0	5 (0.03)	238 (1.47)	47.6×
LSY678	2.5	99 (0.61)	704 (4.37)	7.1×
LSY678	5.0	204 (1.26)	1406 (8.73)	6.8×
LSY678	7.5	69 (0.42)	998 (6.20)	14.5×
LSY678	10.0	19 (0.12)	261 (1.62)	13.7×

Strain LSY678 (10^5 cells) containing plasmid pAURHyg(rep)eGFP was electroporated with varying amounts of the indicated vector. Average colony count from these experiments is presented as well as hygromycin-resistant colonies per aureobasidin-resistant colonies (parentheses). (Four experiments with a variation of ±10%.)

optimal dosage effect may reflect a length preference for DNA pairing events.

Strand bias in gene repair

Evidence has been presented that single-stranded DNA targeting vectors exhibit a strand bias; vectors correct mutations at different, higher frequency dependent on which strand of the helix is targeted. The difference reported by Yoon and colleagues (26) exceeds 1000-fold, remarkably. This characteristic, if true, would provide a strong foundation for predicting correction frequency. The system used here, and our vector designs, allowed us to test this reaction parameter directly. Vectors Hyg3S/74T and Hyg3S/74NT target the transcribed (T) and non-transcribed (NT) strands of the fusion gene, respectively. Five micrograms (of either vector) was electroporated into *LSY678* containing pAURHyg(rep)eGFP and the cells selected in hygromycin. As presented in Table 2, vector Hyg3S/74NT exhibited a 5–6-fold increase in correction efficiency. While these results indicate strand bias (non-transcribed being higher), we have not been able to confirm the 1000-fold difference (26).

Gene repair of a frameshift (insertion) mutation

Vectors Hyg3S/74T and Hyg3S/74NT have demonstrated utility in directing gene repair by nucleotide exchange of point mutations. We next tested the same vector for the capacity to repair a frameshift mutation containing a single-base insertion. To maintain consistency and reduce the potential for sequence-context effects, the mutation was made at the same site in the hygromycin gene where the point mutation is located (Fig. 4A). In this case, the insertion was created by introducing an *A* residue into a glutamine codon, shifting the reading frame and eliminating the expression of the fusion gene.

Table 3. Repair of a deletion mutation by Hyg3S/74T or Hyg3S/74NT

Strain	Vector	Amount (μg)	Average colony count
LSY678	–	0	0
LSY678	Hyg3S/74NT	1.0	1 (0.01)
LSY678	Hyg3S/74NT	2.5	68 (0.42)
LSY678	Hyg3S/74NT	5.0	308 (1.91)
LSY678	Hyg3S/74NT	7.5	276 (1.71)
LSY678	Hyg3S/74NT	10.0	137 (0.85)
LSY678	Hyg3S/74T	1.0	1 (0.01)
LSY678	Hyg3S/74T	2.5	18 (0.11)
LSY678	Hyg3S/74T	5.0	70 (0.43)
LSY678	Hyg3S/74T	7.5	47 (0.29)
LSY678	Hyg3S/74T	10.0	11 (0.07)

Strain LSY678 containing plasmid pAURHyg(del)eGFP was electroporated with the indicated vector at the designated amounts. Average hygromycin-resistant colony counts are presented from a total of three experiments. Numbers in parentheses indicate hygromycin-resistant colonies per 10^5 aureobasidin-resistant colonies. (Three experiments with a variation of ±10%.)

Following the same protocol used for correction of the point mutation, vector Hyg3S/74T or Hyg3S/74NT was electroporated into strain *LSY678*. Rescue of the insertion mutation through gene repair was facilitated by both vectors and pictures of the resistant colonies on plates are presented in Figure 4B. In this experiment, cells containing plasmid pAURHyg(ins)eGFP were treated with 5 μg of vector and, consistent with previous data, no colonies grow under selection unless the correct vector is introduced. The addition of the non-specific vector Kan3S/70T produced no detectable resistant colonies, whereas both Hyg3S/74T and Hyg3S/74NT exhibited a dose-dependent gene repair activity (Fig. 4C). In comparison to the repair activity of a point mutation, correction of the insertion is lower, a reduction of ~5-fold. Again, however, a strand bias for correction is observed; targeting the non-transcribed strand results in a higher frequency of repair as judged by the appearance of hygromycin-resistant colonies.

Previous work from our laboratory (4,12) indicated that the repair of a frameshift-deletion mutation was 10–20-fold lower than the repair of a point mutation, using similar vectors. Other reports indicated high efficiencies of repair of frameshift (deletion) mutation *in vivo* (15). Because Hyg3S/74T and Hyg3S/74NT are capable of directing the correction of a deletion mutation in the hygromycin gene, we created a single deletion mutant in plasmid pAURHyg(+)eGFP generating pAURHyg(del)eGFP. This plasmid was propagated in *LYS678* and targeted for correction by Hyg3S/74T or Hyg3S/74NT. As shown in Table 3, both vectors repair the deletion in a

Figure 3. (Previous page) (**A**) Dose response of gene repair of Hyg3S/74NT for replacement mutation. Different amounts of Hyg3S/74NT (0, 1.0, 2.5, 5.0, 7.5 and 10.0 μg) were electroporated into *S.cerevisiae* (*LSY678)* containing pAURHyg(rep)eGFP. After a recovery of 6 h, 200 μl of yeast cells were spread on hygromycin selective-YPD agar plates and cultured at 30°C for 3 days. The number indicated in the plates was the average number of colonies from four separate experiments. (**B**) The DNA sequence of the vectors Hyg3S/25NT, Hyg3S/40NT, Hyg3S/74NT and Hyg3S/100NT are shown. The table indicates the numbers of hygromycin-resistant colonies obtained after introduction of each vector into *LSY678* containing pAURHyg(rep)eGFP. Strain *LSY678* (10^5 cells) containing plasmid pAURHyg (rep)eGFP was electroporated with equimolar amounts of the indicated vectors. The length of each vector is presented as the number preceding the 'NT' in the name. Average colony counts [see (A)] are outlined, as well as the number of hygromycin-resistant colonies per aureobasidin-resistant colonies.

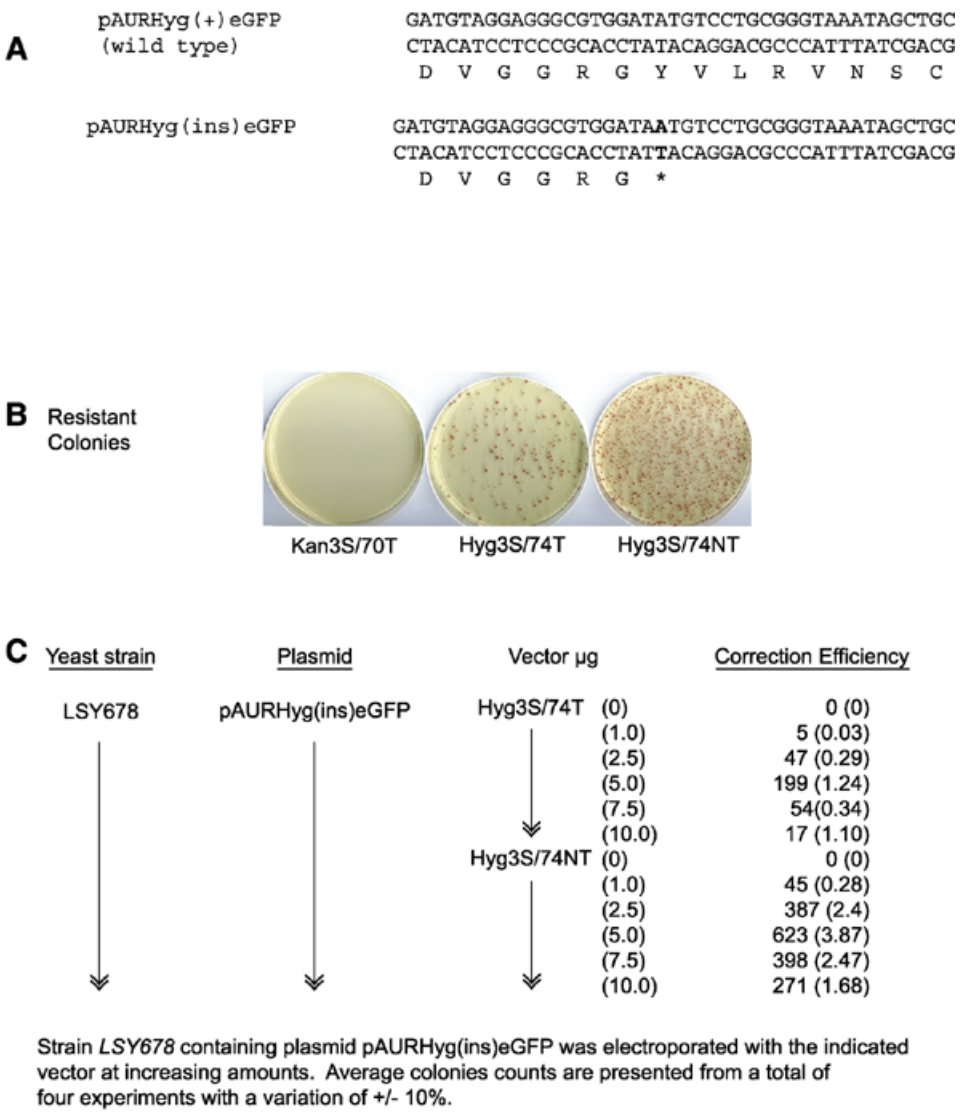


Figure 4. Gene repair efficiency of insertion mutation by Hyg3S/74T and Hyg3S/74NT. (**A**) Five micrograms of oligonucleotide vectors was utilized unless specifically indicated. Kan3S/70T, Hyg3S/74T or Hyg3S/74NT were electroporated into *S.cerevisiae* (*LSY678 mat*α) containing pAURHyg(ins)eGFP, spread on hygromycin-YPD agar plates and cultured at 30°C for 3 days. The mutant is created by a single base (A) insertion between nucleotides 136 and 137 of the hygromycin B gene in plasmid pAURHyg(ins)eGFP (symbolized as *). (**B**) One of four separate experiments showing comparative gene repair between Hyg3S/74T and Hyg3S/74NT in *LSY678* matα with pAURHyg(ins)eGFP. The number of hygromycin-resistant colonies were 0 (left), 72 (middle) and 1462 (right) which were targeted with Kan3S/70T, Hyg3S/74T and Hyg3S/74NT, respectively. (**C**) *LSY678* matα containing pAURHyg(ins)eGFP were electroporated with the indicated vectors at increasing amounts. Average colonies counts are presented from a total of four separate experiments with a variation ±10%.

dose-dependent fashion but at different frequencies. Hyg3S/74NT corrects at a 10-fold higher frequency than does Hyg3S/74T, again revealing a strong strand bias. In agreement with our earlier data (4,11), however, the repair efficiency is substantially lower than repair of a point mutation and is also lower than the correction of a frameshift-insertion mutation.

Phenotypic expression of altered genes can also be examined by checking for the presence of green fluorescence since the target gene is a fusion of hygromycin and eGFP; the appearance of green fluorescence can be monitored by laser confocal microscopy (2,23). Yeast cells (*LSY678*) were electroporated with Hyg3S/74NT and the cells processed for evaluation by confocal microscopy. Figure 5 illustrates expression of a corrected fusion gene visualized by GFP fluorescence. Panels A and E depict cells containing the wild-type plasmid while B exhibits cells containing pAURHyg(rep)eGFP. Panel C depicts cells containing the mutant plasmid, pAURHyg(ins)eGFP, and D illustrates cells containing the mutant plasmid pAURHyg(del)eGFP. Panels F, G and H illustrate cells containing the mutant plasmid from B, C and D, respectively, except that Hyg3S/74NT has been electroporated into each strain. In panels A and E, green fluorescence is observed as a function of wild-type plasmid expression. In B, C and D, a low level of autofluorescence is observed in the mutant cells, but expression of the wild-type eGFP fusion product is evident only in F, G and H. The extent of eGFP appearance is maximized because these cells have been pre-selected with hygromycin. These results correspond to those obtained using

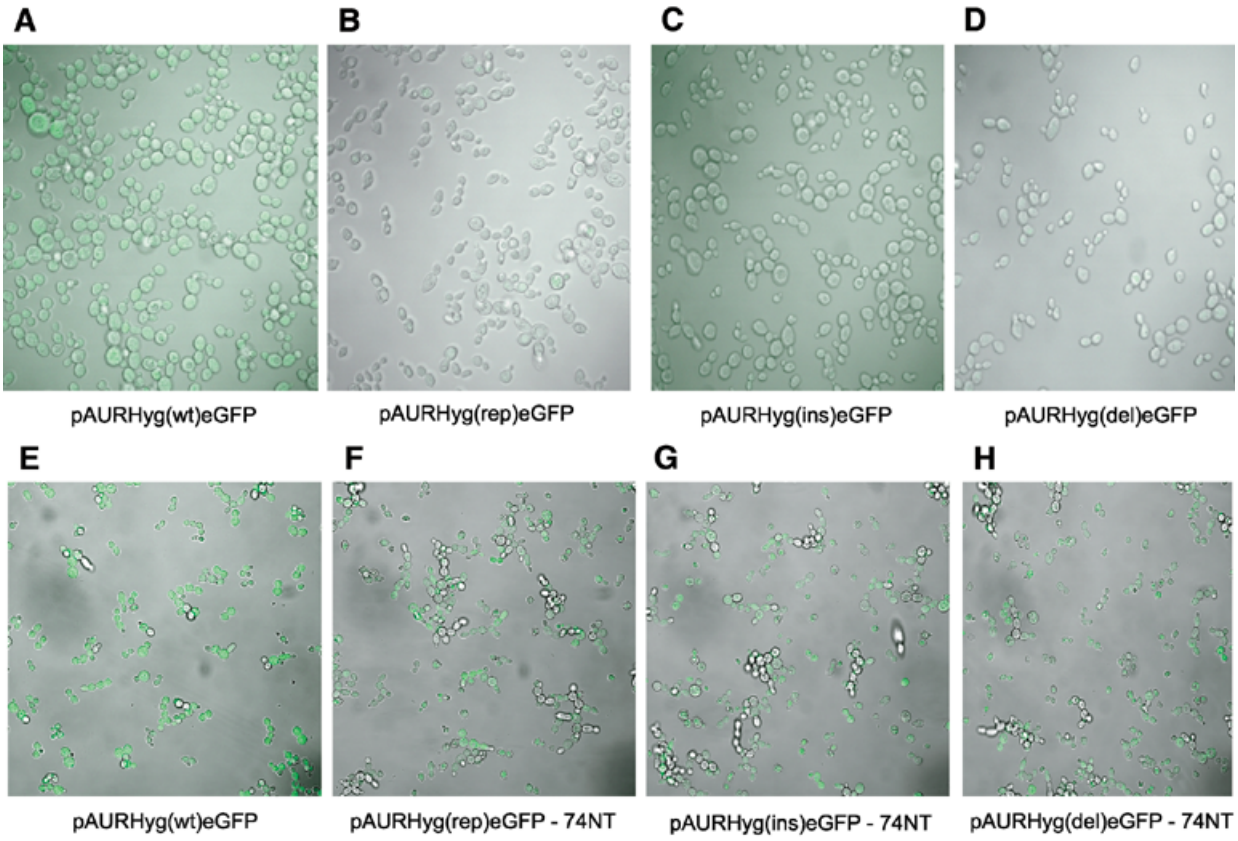


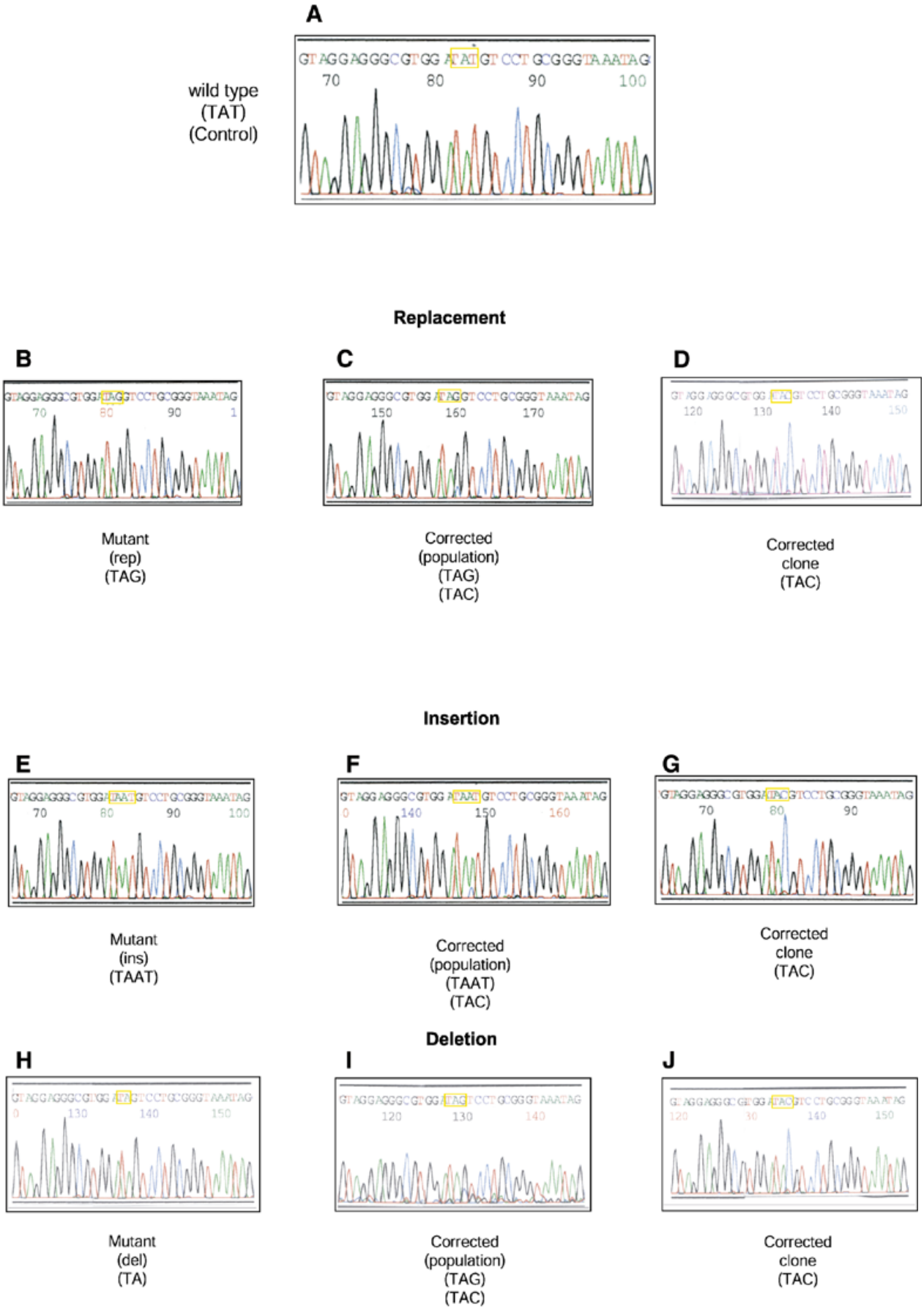
Figure 5. GFP expression. Single hygromycin-resistant colonies were picked and cultured in YPD media for 1–3 h. The expression of eGFP was visualized with A Zeiss LSM 510 confocal microscope using a 505–550 nm bandpass. (**A**) and (**E**) present the expression of GFP in *LSY678* matα containing pAURHyg(wt)eGFP without or with the electroporation of the non-specific oligonucleotide Kan3S/70T. An image of untransfected *LSY678* matα bearing pAURHyg(rep)eGFP, pAURHyg(ins)eGFP and pAURHyg(del)eGFP shows minimal background fluorescence, as pictured in (**B**), (**C**) and (**D**), respectively. The expression of GFP was observed in Hyg3S/74NT-transfected *LSY678* matα harboring pAURHyg(rep)eGFP, pAURHyg(ins)eGFP and pAURHyg(del)eGFP as represented IN (**F**), (**G**) and (**H**), respectively.

hygromycin selection as the detection assay for gene repair. Hence, nucleotide repair directed by these oligonucleotides can be measured phenotypically through the expression of green fluorescent protein as well as by antibiotic selection.

A DNA sequence analysis was performed to confirm gene repair at the nucleotide level. As shown in Figure 6, PCR products generated from isolated plasmid DNA from replacement, insertion and deletion mutants revealed mixed peaks at the position of the targeted nucleotide. Isolation of plasmid DNA originating in hygromycin-resistant yeast colonies and propagated in *E.coli* revealed a pure DNA sequence containing the corrected base at the specific position. This indicates that a subset of plasmid molecules are converted. As described earlier, directed repair is from TA*G* to TA*C* not TA*T* to avoid concerns of wild-type plasmid contamination. Sequence analyses from 10 hygromycin-resistant colonies revealed only TA*C* codons at the specific site reflecting the precision of the repair event (third panel of replacement group). In the case of the insertion, however, the TA*T* wild-type codon has been altered to TA*A*T yet the corrected plasmids had a sequence of TA*C*. While this is the specified base repair event, the T residue 3′ to the targeted base has been removed to generate the pure population of TA*C* (third panel of insertion group; Fig. 6). The second panel is a DNA sequence obtained from hygromycin-resistant colonies in total while the third panel of DNA sequences originates from individual colonies propagated in *E.coli*. Thus, in the case of the repair of an insertion mutation, two bases (A and T) are removed and one C is inserted. Repair of the deletion mutation exhibits a similar pattern. Taken together, these data confirm gene repair at the nucleotide level and indicate that not all plasmid targets in resistant yeast cells are corrected.

DISCUSSION

Synthetic polynucleotides can be used as vectors to direct single base changes in yeast. Pioneering work by Sherman and colleagues (24) demonstrated the correction of a point mutation in an episomal target, work extended by Yamamoto *et al.* (25). In this study, we have taken a similar approach to mutation correction using oligonucleotides that have been shown to mediate gene repair in mammalian cells and plants (2 and references therein). The target is a fusion gene, constructed by merging two phenotypic readouts, hygromycin resistance and green fluorescence. The construct contains a mutation in the region of the gene conferring hygromycin


A
wild type
(TAT)
(Control)
Replacement
B
Mutant
(rep)
(TAG)
C
Corrected
(population)
(TAG)
(TAC)
D
Corrected
clone
(TAC)
Insertion
E
Mutant
(ins)
(TAAT)
F
Corrected
(population)
(TAAT)
(TAC)
G
Corrected
clone
(TAC)
Deletion
H
Mutant
(del)
(TA)
I
Corrected
(population)
(TAG)
(TAC)
J
Corrected
clone
(TAC)

resistance in yeast, hence, directed gene repair enables both resistance to hygromycin and expression of green fluorescence. The assay system is constructed so that the plasmid construct resides in low copy number and, thus, the frequency of gene repair can be estimated with greater confidence by quantifying hygromycin-resistant colonies.

We report that modified single-stranded DNA oligonucleotides (11,12) direct gene repair of point and frameshift mutations, but do so with differing efficiencies. Our data suggest that (i) a point or replacement mutation is corrected by vectors having a length between 25 and 100 nt, respectively, with a 74mer being the most active; (ii) frameshift mutations, including both insertion and deletion, are repaired less efficiently—the repair of an insertion being 5-fold better than a deletion. An order of repair efficiency can be tentatively envisaged as replacement > insertion > deletion, respectively; (iii) the repair of each type of mutation can be observed through the appearance of either hygromycin-resistant colonies or green fluorescence or both; (iv) the repair of all three mutations occurs in a dose-dependent manner with an optional concentration being realized at ~5.0 μg/10^5 cells; (v) the same strand bias exists for the repair of each type of mutation, targeting the non-transcribed (sense) strand results in higher colony counts than targeting the transcribed (antisense) strand; (vi) each resistant colony processed for DNA sequencing revealed a population of isolated plasmids that contained the specified base alteration. Hence, these results indicate that some plasmid targets in hygromycin-resistant colonies remain uncorrected; and (vii) the same targeting vector can direct the repair of point, insertion and deletion mutations and that the correction of the insertion results in a multiple nucleotide rearrangement. Here, two nucleotides are removed while one is inserted. (viii) There is an optimal nucleotide length for vectors active in gene repair.

In previous studies, Gamper *et al.* (12) identified the active 'repair-domain' of the CO as the region of uninterrupted DNA, prompting the development of the new repair vectors used in this study. Cell-free extract experiments then confirmed that these specifically modified single-stranded DNA vectors direct gene repair with high efficiency. Rice *et al.* (2) extended these observations *in vivo* by exploiting *S.cerevisiae* as a model system and identified several genes, which regulate gene repair. Gamper *et al.* (11) also showed that increasing the length of the chimera elevates gene repair activity *in vitro*. We extend those studies here *in vivo* and report that an optimal length for gene repair activity exists among the single-stranded molecules. In this system the optimum appears to be between 74 and 100 nt. The decrease observed when Hyg3S/100NT is used is likely due to the development of secondary structure which may reduce DNA recombinase activity.

In fact, the higher levels of targeting observed by Gamper *et al.* (11) when the chimera was used probably reflects the increasing length of the DNA strand of the double hairpin molecule. Since we showed earlier that this strand directs correction (12), the longest uninterrupted DNA section of all the chimeras tested was 30 bases, aligning closely with data obtained here using Hyg3S/25NT. Synthesis of chimeric RNA/DNA nucleotides with DNA stretches of 70–80 bases in length may elevate chimera-directed gene repair. Other laboratories have also employed single-stranded molecules for targeting experiments in various systems (26,27) supporting their use in genetic manipulation. Recently, Parekh-Olmedo *et al.* (28) demonstrated targeted gene repair in HeLa cells using modified single-stranded vectors, outlining a simple protocol for assessing activity.

One significant aspect of the current study is that it features yeast as the model organism for testing gene repair vectors. Because the *S.cerevisiae* is a genetically tractable organism, elucidation of the regulatory circuitry of gene repair is enabled. In addition, considering the conservation of DNA repair protein sequence and function from yeast to human, it is likely that homologs of the yeast proteins involved in repair will be active in experiments of targeted repair in human cells. Thus far, RAD52, RAD51, MSH2, MSH3 and RAD10 are among those genes already identified to be participating in the reaction (23). The advantages of using yeast as a model system for synthetic vector-directed gene repair were recognized earlier by Sherman and colleagues (24,25) as described above.

The data reported herein establishes that, for the first time, a single molecular structure, Hyg3S/74 (T or NT), can direct the repair of point and frameshift mutations and that the genetic changes are stably inherited. Vectors designed as a sequence compliment of the non-transcribed strand afford a higher level of repair. Recently, Igoucheva *et al.* (26) reported strand bias in experiments in CHO cells at a 1000-fold higher frequency when the non-transcribed (template) or sense strand was targeted. Our results, while supporting strand bias, cannot confirm the 1000-fold difference; in fact, we observe only a 5–50-fold enhancement.

Moerschell *et al.* (24) described a system in which the genomic copy of the *cyc1* was altered with synthetic oligonucleotides. However, additional changes were observed at nearby sites perhaps due to the level of oligonucleotides transformed into the yeast cells (100 μg). These studies extended earlier work by Burke and Olson (29), Walder and Walder (30) and Mandecki (31) wherein oligonucleotides were employed to direct nucleotide alteration in *E.coli*. Our data agree in part with these pioneering efforts, but differences do exist. First, successful correction of mutations in the hygromycin gene was observed at lower levels of transformed oligonucleotides (1–10 μg). This difference between the results is most clearly

Figure 6. (Previous page) Sequence confirmation of the hygromycin gene correction in *LSY678* matα containing pAURHyg(rep)eGFP, pAURHyg(ins)eGFP and pAURHyg(del)eGFP. Single hygromycin resistance colonies were picked for colony PCR, and the PCR products were sequenced, as described in Materials and Methods. Meanwhile, plasmids were rescued from hygromycin-resistant colonies and transformed into *E.coli*, isolated by mini-prep kits as described in Materials and Methods. (**A**) PCR product amplified from *LSY678* matα containing pAURHyg(wt)eGFP showing the sequence of TA*T* at codon 46 of hygromycin B gene (highlighted in yellow). (**B**), (**E**) and (**H**) PCR products were amplified from pAURHyg(rep)eGFP, pAURHyg(ins)eGFP and pAURHyg(del)eGFP, respectively, showing the sequence TA*G*, TA*A*T and TA at the same position within hygromycin B gene prior to addition of the vector. (**C**), (**F**) and (**I**) represent a population of mutant and corrected plasmid samples observed in the same hygromycin-resistant colony isolated from yeast. A mixed population of TA*G*/TA*C*, TA*A*T/TA*C* and TA/TA*C* is shown in (C), (F) and (I), respectively. In (**D**), (**G**) and (**J**) the corrected TA*C* at codon 46 of hygromycin B gene is observed after sequencing of the mini-prep plasmids, isolated from *LSY678* matα containing pAURHyg(rep)eGFP, pAURHyg(ins)eGFP or pAURHyg(del)eGFP and propagated in *E.coli*.

amplified in the toxicity of yeast cells exhibited at even the 10 μg levels (per 10^5 cells) in the current system. Our dose curves indicate that a narrow window of oligonucleotide concentration exists for targeting success. Secondly, the most active oligonucleotides used in this study were found to be 74 nt in length as compared to the 20mers used by Moerschell *et al.* (24). Third, Yamamoto *et al.* (25) observed a radical difference in the targeting frequency of the *cyc1* gene. Sense oligonucleotides, targeting the transcribed strand, exhibit a higher level of correction. The possibility that the sense oligos were acting simply in an antisense fashion, inhibiting translation of the *cyc1* transcript were ruled out by experimentation (25,32). It is also unlikely that the rate of transcription was adversely affected by the 'sense' oligonucleotide. The results reported herein also differ from these data. We define the oligonucleotides based on the target strand within the gene to which they bind. For example, Hyg3S/74T targets the transcribed strand making it equivalent to the 'sense' oligos described by Yamamoto *et al.* (25). As shown in Table 2, etc., Hyg3S/74T is much less effective than Hyg3S/74NT, perhaps the difference lies in the type of target, genomic versus episomal; we are now extending our studies to the genomic level.

In our system, the 'NT' oligonucleotide vectors would be complementary to the mRNA, yet, the NT-vectors are more efficient than T vectors; targeting the non-transcribed strand leads to a higher level of repair. In support of this, a greater degree of mutation has been found to occur on the non-template strand (33–35) during transcription. It is plausible that strand bias is a result of the presence of RNA polymerase or mRNA, which may, either alone, or in combination, reduce accessibility of the target DNA strand. While it is widely accepted that transcribed strands are repaired more efficiently than non-transcribed strands, directed DNA repair is unique and steric hindrance by the so-called 'transcriptosome' (36), in total or in part, may account for the observed strand bias.

Independent of the reaction differences in data, yeast clearly provide an important system in which to examine oligonucleotide-directed gene repair. Subsequent experiments by Yamamoto *et al.* (37) began to define the genes catalyzing this reaction, and our most recent work (23) examines this issue in greater detail. As this field matures and experimental data centered on the mechanism of repair accumulates, a more complete picture will emerge enabling a smooth transition into higher eukaryotes.

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