The involvement of replication in single stranded oligonucleotide-mediated gene repair

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ABSTRACT
Targeted gene repair mediated by single-stranded oligonucleotides (SSOs) has great potential for use in functional genomic studies and gene therapy. Genetic changes have been created using this approach in a number of prokaryotic and eukaryotic systems, including mouse embryonic stem cells. However, the underlying mechanisms remain to be fully established. In one of the current models, the ‘annealing-integration’ model, the SSO anneals to its target locus at the replication fork, serving as a primer for subsequent DNA synthesis mediated by the host replication machinery. Using a λ-Red recombination-based system in the bacterium Escherichia coli, we systematically examined several fundamental premises that form the mechanistic basis of this model. Our results provide direct evidence strongly suggesting that SSO-mediated gene repair is mechanistically linked to the process of DNA replication, and most likely involves a replication intermediate. These findings will help guide future experiments involving SSO-mediated gene repair in mammalian and prokaryotic cells, and suggest several mechanisms by which the efficiencies may be reliably and substantially increased.

INTRODUCTION
During the past few years, targeted genetic modification mediated by single-stranded oligonucleotides (SSOs) has been proven to be a very precise and efficient technique for engineering DNA, especially for functional genomic studies (1–3). Using this approach, it is possible to generate specific changes in essentially any DNA sequence: to create deletions, insertions or point mutations in bacteria, fungi and mammalian cells. The use of SSOs for the creation of specific mutations is ideally suited for the study of single-nucleotide polymorphisms (SNPs) in model organisms such as mice, and ultimately may be used for the treatment of diseases caused by subtle DNA alterations (4,5). Considering the advantages of targeted gene repair using SSOs over other contemporary therapeutic approaches, such as the use of viral vectors, this technology holds great potential to form the basis of future gene therapy-based treatments (6–8).

To date, it has been proposed that SSO-mediated DNA repair may proceed via a number of different mechanistic pathways, including: mismatch repair, where the SSO acts as a template directing the mutagenesis (7); direct incorporation of the SSO through DNA synthesis; as well as other routes requiring proteins and DNA intermediates involved in double strand break repair and homologous recombination (9–11). However, little direct evidence has been reported to support any of these hypotheses, except that SSOs have been observed to be incorporated in to chromosome target in mammalian cells (12). We selected E. coli as a model system within which to study the mechanism of SSO-mediated DNA repair. This was due to the widespread use of SSO-mediated DNA engineering (also referred to as recombiner- ing) in functional genomic studies in this bacterium, as well as the ready availability of other genetic tools (2,13). In addition, there is one critical difference between SSO-mediated gene repair in mammalian cells and in bacterial cells: exogenous proteins such as the Red proteins from λ-phage are necessary for efficient SSO-mediated gene repair in bacteria, whilst only endogenous proteins are involved in similar studies in mammalian cells. We, therefore, investigated whether the direct incorporation of an SSO into its target was a mechanistic pathway that was utilized in bacteria.

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SSOs have recently been used to direct sequence-specific mutations in E.coli with the help of the rac-encoded RecET system or the bacteriophage-λ Red recombination system (14–18). The efficiencies of both these systems are remarkably high, with generally >1% of the SSO-treated cells being recombinants, making it possible to screen for mutants without using a selectable marker (9,19). Using similar methodology, the RecET and λ-Red systems may also be used to create deletions, insertions or substitutions in E.coli, using appropriately-designed PCR-generated DNA fragments, eliminating the need for in vitro manipulations using restriction enzymes or DNA ligases (2,13). Due to it being better characterized and more widely used than RecET, we have focused our studies on the λ-Red system (20).

The three proteins of the Red system: Exo, Beta and Gam mediate recombination between a linear double-stranded DNA (dsDNA) donor and its homologous target sequence by promoting double strand break repair (DSBR) (21–23). Exo is a dsDNA exonuclease that digests linear DNA fragments in the 5’→3’ direction, generating two 3’-overhangs that act as substrates for recombination at each end (24). Beta is a single-stranded DNA binding protein that promotes the annealing of complementary single strands, whilst the Gam protein inhibits the host RecBCD nuclease (27). Mutational analysis has indicated that Beta is the only Red protein that is absolutely required for SSO activity, with the deletion of both exo and gam causing only a minor decrease in recombination efficiency.

Court et al. (9) previously proposed an ‘annealing-integration’ model, in which the SSO first anneals to a single-stranded region at the replication fork, before DNA polymerase and ligase complete the gene repair process by extending the annealed SSO and joining it to the chromosomal or plasmid DNA. Consistent with this model, it has been observed that two correction-SSOs with complementary sequences (hence targeted to the same chromosomal location) had different correction efficiencies (9,20). The SSO with the same sequence as the nascent lagging strand (in DNA replication) was found to be more efficient at mediating targeted gene repair than the other. This strand bias may be explained by the presence of more single-stranded regions of DNA during lagging strand synthesis, which the SSO may access, compared with during leading strand synthesis.

Several fundamental premises form the mechanistic basis of the ‘annealing-integration’ model. Firstly, the model implies that the SSO is annealed and subsequently incorporated into its dsDNA target. Secondly, the SSO is incorporated into a nascent strand, most probably by serving as a primer for host replication. As the SSO is predicted to anneal to its transiently single-stranded target at the replication fork, such gene repair processes presumably require active replication of the target DNA molecule. Consequently, mutations in host factors that are involved in DNA replication should alter the frequency of gene repair events. Finally, the model also implies a minimal requirement for host recombination factors that are essential for conventional homologous recombination in the bacteria. To gain further insight into the mechanistic basis of SSO-mediated gene repair in E.coli, we decided to investigate the plausibility of the ‘annealing-integration’ model by experimentally verifying or disproving each of the above premises.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides (SSOs, oligos) were purchased in a desalted form without further purification (Sigma-Proligo, Singapore). Sequences of correction-SSOs for the mutant chloramphenicol antibiotic reporter are listed in Supplementary Material Table 6. SSOs containing bases connected via phosphothioate (PT) groups and/or a 3’-terminal dideoxycytidine were purchased from Sigma-Proligo (Singapore). In experiments conducted with the mutant kanamycin reporters, a 91 nt SSO (oligo 12A) was used.

Genotype of strains

All strains used in this study were derived from DY380 which harbors the λ Red genes and are recA defective (15). Normally, 10–50% of bacterial cells survived electroporation and the various strains exhibited similar survival rates either when electroporated with or without a correction-SSO (data not shown). Plasmids were propagated in DH10B (F-mcrAΔ(mrr-hsdRMS-mcrBC)Φ80dlacZΔ M15 ΔlacX7 deoR recA1 endA1 araΔ1397 ara, leu)7649galUgalKrspl-nupG]. Genotypes of experimental strains are listed in Supplementary Material Table 7.

Plasmid construction

DNA inserts for plasmid constructs were amplified by PCR using the Expand High Fidelity PCR system (Roche) and primers linked to restriction enzyme sites suitable for cloning. Inserts were routinely sequenced to check for possible mutations introduced by PCR. Plasmid DNA was isolated using Qiagen mini kits (Qiagen GmbH, Germany). For plasmid maintenance, kanamycin (kan), chloramphenicol (cm), tetracycline (tet) and/or ampicillin (amp) was added to Luria–Bertani medium (LB medium) at 50, 25, 12.5 and 50 μg ml⁻¹, respectively.

pmKan Primers mkan-1 and mkan-2 were used to amplify a 559 bp fragment of plasmid PGK-frt (courtesy of Dr A.F. Stewart). The Eagl and Ncol digested 559 bp PCR fragment was purified then ligated into the 4287 bp Eagl–Ncol fragment of PGK-frt to form pmKan.

p"mKan pBluescript KS" (Stratagene) and pmKan were digested with EcoRI and Clal. The 1858 and 2940 bp DNA fragments were recovered from their respective plasmids and ligated to generate pBS-mKan. The 705-flp plasmid (containing the pSC101 temperature-sensitive replication origin, courtesy of Dr A. F. Stewart) and pBS-mKan were digested with Clal and Sacl. The 1813 and 6444 bp fragments were recovered from their respective plasmids and ligated to form p°mKan.

Verification of biotinylated-SSO incorporation

λ-Red expression was induced in cultures of DY380/pmKan cells (in 50 ml LB medium in 250 ml Erlenmeyer flasks, grown to OD₆₀₀ 0.3) by shifting them from incubation at 32°C to a shaking water bath at 42°C for 15 min, before chilling them in iced-water. After making the cells electrocompetent, essentially as described previously (20), aliquots (50 μl) were electroporated with the biotinylated oligo B-12A (100 ng). Cells were then allowed to recover by...
resuspending them in LB medium (1 ml), followed by incubation at 32°C for 20 min with gentle shaking. After isolation of the plasmid DNA, biotinylated DNA was captured on streptavidin-conjugated magnetic agarose beads (Novagen), and was purified as described by Edwards et al. (28). The identity of the biotinylated pmKan plasmid was confirmed by PCR amplification of a 496 bp-region of the kan gene which contained the site of the original point mutation using primer 1 and primer 2.

Suppression of DNA replication

Overnight cultures of DY380/p²mKan or DY380/pmKan were diluted 50 times in fresh LB medium, extended until OD₆₀₀ = 0.6 at 32°C, then transferred to 42°C for 15 min to induce Red expression. The cells were chilled and subsequently electroporated with oligo 12A (100 ng) as described above, then LB media (1 ml) was added. Equal aliquots of the electroporated DY380/p²mKan and DY380/pmKan cells were incubated at 37°C in a water bath with shaking for 0, 15, 30, 60 or 120 min to suppress plasmid replication, followed by an additional incubation at 32°C for 120, 105, 90, 60 or 0 min, respectively. Aliquots of the bacterial cells were separately spread onto LB-media agar plates with kanamycin and/or ampicillin, and incubated at 32°C overnight.

Generating the mutant chloramphenicol antibiotic gene reporter allele

The chloramphenicol acetyltransferase (CAT) point-mutant allele was generated by PCR-site directed mutagenesis using oligos mcm1 and mcm2 that changed the TAC to a TAG amber codon. The PCR product was digested with EcoRI and SpeI to replace the original sequence of the 5’CAT gene on a pBluescriptIIKS(+) plasmid. The fragment carrying the mutant CAT gene was released by EcoRV digestion and was blunt-end ligated to the pGK-frt plasmid (Courtesy of Dr F. Stewart) after treating the vector with ClaI and T4 polymerase to generate pGKfrtmCM(+) and pGKfrtmCM(–). These reporter plasmids are identical except for the orientation of the mutant antibiotic gene allele relative to the ColE1 replication origin.

To assay gene repair events between the SSO and its chromosomal target, the mutant reporter (mCM) was integrated into the DY380 chromosome as described previously (20) in both orientations relative to oriC (Figure 1), using oligo mcm(+)F and mcm(–)F as forward primers to generate DY380mCM(+) and DY380mCM(–) respectively, and mcmB as reverse primer for both reporter strains. PCR analysis was performed to identify clones with the reporter allele correctly inserted at both the 5′ and 3′ integration sites, and positive clones were subsequently sequenced. The kanamycin resistance gene flanked by frr sites was removed by transforming and inducing Flp expression carried on the 705-flp plasmid (Courtesy of Dr A. F. Stewart), to yield the reporter strain with a ca. 100 bp insertion in the mCm gene at the site where the original point mutation was located [DY380mCM(+)loxP].

Generating mutant derivatives

Using recombinase PCR-amplified targeting cassettes containing a selectable marker with flanking homology to the appropriate target sequences, were used to generate several null alleles including dnaQ, holC, holD and mutS, as well as a number of double mutants. The ssb113 allele was generated using a similar approach: with a single base mutation incorporated into the forward primer used for amplifying the PCR targeting cassette. The ssb113 allele was sequenced after PCR analysis, to confirm that only the desired mutation had been introduced. The oligos used for the construction of each strain are described in detail in Supplementary Material Table 6. Several independent clones of each mutant were assayed to avoid potential artifacts caused by suppressor mutations.

Targeted gene repair assays using a mutant antibiotic reporter system

DY380 and derivatives were grown and induced as described previously (20). A saturating amount of SSO (100 ng) was subsequently removed by transforming and inducing Cre expression carried on the 705-cre plasmid (Courtesy of Dr A. F. Stewart), to yield the reporter strain with a ca. 100 bp insertion in the mCm gene at the site where the original point mutation was located [DY380mCM(+)loxP].
introduced via electroporation and the cells were allowed to recover in 1 ml LB at 32°C for 2 h. Thereafter, the cells were plated onto LB agar plates and LB agar supplemented with 12.5 μg ml⁻¹ chloramphenicol. Gene repair efficiencies were calculated by dividing the number of colonies that formed after 24 h at 32°C on the antibiotic-containing plates by the number that appeared on the LB agar plates without antibiotic. Results were quoted as the mean (±SD) percentage of colonies that were chloramphenicol resistant (CM'), from at least three individual experiments, each performed in triplicate. No observable differences in transformation efficiencies (>90%) of the various strains were detected when a fluorescein-labeled oligo was introduced following identical procedures.

The spontaneous formation of CM' colonies for each strain was also examined using the procedures described above, using mock electroporation (i.e. without SSO) of the cells.

**Cell viability of mutant strains**

Cell viability was assayed by calculating the number of colony forming units of the bacterial cells during growth at exponential phase. Overnight cultures inoculated from a single colony of each strain were diluted 50 times into LB media and allowed to grow until reaching an OD₆₀₀nm of ~0.5. Thereafter, appropriate dilutions of the cells were plated onto LB agar plates and incubated at 32°C for 48 h.

**Data analysis**

The proportion of cells that underwent gene repair events (quoted as a percentage) in the various reporter strains, under different treatment conditions etc., were subjected to student’s t-tests to evaluate their significance relative to the appropriate controls. Significance levels were set at 0.05.

**RESULTS**

The mechanistic basis of SSO-mediated gene repair, in *E.coli*, as well as other organisms, remains largely speculative in nature. According to one of the current models, the ‘annealing-integration’ model, the correction-SSO anneals to its target locus at the replication fork, such that the host replication machinery incorporates the SSO as a nascent strand during DNA replication.

**Direct incorporation of correction-SSO**

To investigate whether or not the correction-SSO was directly incorporated into its dsDNA target, we used a biotinylated SSO to target a plasmid-based mutant antibiotic gene employing a similar protocol as described previously (12). Consequently, the successful integration of the biotinylated SSO into the ‘repaired’ plasmid could be confirmed by using streptavidin beads to isolate only biotinylated plasmid DNA. Using standard cloning techniques, a plasmid (pmKan) was constructed that harbored a kanamycin resistance gene (*kan*), which had been engineered to contain a nonsense mutation in its coding sequence (Y22Stop) as previously described (20). pmKan was transformed into *E.coli* DY380 (29), a strain that contains a chromosomal copy of the λ Red system (i.e. the *exo, bet* and *gam* genes) under the strict control of the thermosensitive cI857 repressor. The nonsense mutation in the *kan* gene meant that cells transformed with pmKan remained sensitive to kanamycin. A 91-mer correction-SSO (oligo 12A) was designed to convert the nonsense mutation (TAG) in the *kan* gene to a new silent mutation (TAC), restoring the gene function. The corrected pmKan DNA was isolated from individual revertant colonies and was sequenced to confirm that only the intended changes had been introduced.

We first established that a correction-SSO which had been internally labeled with two biotin moieties (oligo B-12A) had a similar correction efficiency to that of an unlabeled SSO of identical sequence (oligo 12A). Both SSOs were found to have similar efficiencies of ca 20% (data not shown). Plasmid DNA was purified from the lysates of λ Red-induced DY380/ pmKan cells which had been transformed with the biotin-labeled oligo B-12A or the unmodified oligo 12A (for schematic see Figure 2A). Biotinylated DNA was isolated by adsorption onto streptavidin-conjugated magnetic agarose beads, which were then extensively washed to remove traces of DNA that had bound non-specifically. After release of the biotin-labeled DNA from the beads by denaturation of the streptavidin protein, the presence of the pmKan plasmid was confirmed by PCR amplification of a 496 bp section of the *kan* gene that contained the targeted change, followed by electrophoretic analysis on an agarose gel. As shown in Figure 2B, the retention of pmKan DNA on the streptavidin beads depended upon both the activation of Red-mediated gene repair activities, and upon the presence of the biotinylated correction-SSO. Biotinylated plasmid DNA could be detected as early as 15 min after transformation. These results demonstrated that during the SSO-mediated gene repair event, the biotin-labeled SSO had become physically incorporated into the reporter plasmid, resulting in its biotinylation.

**Extension of the SSO is necessary for efficient gene repair**

To test whether the correction-SSO served as a primer for DNA synthesis, we designed a 89mer that contained a central mismatch, with flanking sequences homologous to the point-mutant kanamycin reporter gene, as well as a chain-terminating 2',3'-dideoxyctydine (ddC) residue (PT-ddC SSO). The SSO was stabilized from exonuclease cleavage by incorporating PT linkages at both termini. An analogous SSO that contained similarly positioned PT linkages but a 3' terminal dideoxyctydine residue (PT SSO), served as a control. We first confirmed in a PCR reaction (which included mCM(+)DT2 as the reverse primer) that the dideoxyterminating SSO (PT-ddC SSO) was sufficient to inhibit extension by a DNA polymerase endowed with 3' exonuclease proofreading activity (Figure 3), while the SSO containing PT linkages exclusively (PT SSO) can prime DNA synthesis in an *in vitro* PCR reaction.

A pair of plasmids, pGKfrtmCM(+) and pGKfrtmCM(−), were constructed for use as reporters to study the efficiency of gene repair mediated by correction-SSOs that contained chain-terminating ddC nucleotides. Both reporter plasmids harbored a defective chloramphenicol resistance gene (mCM) where a tyrosine-encoding codon (TAC) had been replaced with an amber stop codon (TAG). Accurate correction of this antibiotic allele conferred chloramphenicol resistance to the reporter bacteria. After transformation of the plasmid into Red-positive *E.coli* strains, the biotin-labeled correction-SSO was found to prime DNA synthesis, and subsequently the pmKan plasmid was sequenced, demonstrating that the corrected antibiotic resistance gene was restored.
resistance to the host. The two plasmid reporters were identical except for the orientation of the mCM gene, with respect to the ColE1 replication origin (+ or /C0). The plasmid substrates were first established as stable episomes in DY380. Subsequently, the DY380/pGKfrtmCM cells were induced for λ-Red expression prior to transformation with either the PT+ddC or PT SSOs. As predicted by the ‘annealing-integration’ model, the PT+ddC SSO directed gene repair events at reduced efficiencies at all three concentrations tested, when compared to the levels directed by the PT SSO (Table 1).

To confirm that the direct extension of a correction-SSO was also important for efficient chromosomal gene repair, the point-mutant chloramphenicol antibiotic gene (mCM) was integrated into the bioA locus on the chromosome of DY380, in both orientations relative to the replication origin (oriC), to generate the reporter strains DY380mCM(+/C0) and DY380mCM(+/+). After induction of λ-Red functions, the reporter strains were electroporated with either the ddC+PT or PT SSOs. By using two oppositely orientated reporter genes, the correction-SSOs corresponded to the sequence of either the nascent leading strand, as in DY380mCM(+/C0), or the nascent lagging strand, as in DY380mCM(+/+). As shown in Table 1, the use of the PT+ddC SSO resulted in significant reductions in gene repair efficiencies in both reporter strains, when compared with the PT SSO. Moreover, consistent with the results from the analogous experiments using the plasmid-based reporter gene, the reductions in repair efficiency were more pronounced when lower concentrations of SSO were used, i.e. 10 ng compared to 100 ng. We attributed this effect to slight heterogeneities in composition of the PT+ddC SSO species present within the cell (further discussed below). The residual gene correction activity observed for the PT+ddC SSO may be due to the presence of small amounts of SSOs that are slightly truncated. These may have originated from errors during the solid-phase synthesis of the SSO, or may be due to partial digestion or an internal cleavage of the SSO in vivo. This would allow DNA extension by the host replication machinery to complete the repair process. However, we cannot exclude the possibility that an additional (minor) pathway is also operating, where gene repair does not require extension of the correction-SSO at the 3-terminus.
Table 1. Extension of mutagenic SSOs is important for efficient gene repair

<table>
<thead>
<tr>
<th>Amount of SSO</th>
<th>Correction efficiency (%) using PT SSO</th>
<th>Correction efficiency (%) using PT+ddC SSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>DY380/pGKfrt(CM+) reporter strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 ng</td>
<td>6.10 ± 2.57</td>
<td>4.03 ± 1.16*</td>
</tr>
<tr>
<td>10 ng</td>
<td>5.76 ± 2.18</td>
<td>0.58 ± 0.46**</td>
</tr>
<tr>
<td>1 ng</td>
<td>0.35 ± 0.19</td>
<td>0.06 ± 0.03**</td>
</tr>
<tr>
<td>DY380/pGKfrt(CM−) reporter strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 ng</td>
<td>4.00 ± 2.52</td>
<td>2.65 ± 2.84</td>
</tr>
<tr>
<td>10 ng</td>
<td>1.63 ± 0.78</td>
<td>0.29 ± 0.12**</td>
</tr>
<tr>
<td>1 ng</td>
<td>0.17 ± 0.12</td>
<td>0.005 ± 0.004**</td>
</tr>
<tr>
<td>DY380mCM(+) reporter strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 ng</td>
<td>1.10 ± 0.45</td>
<td>0.21 ± 0.15*</td>
</tr>
<tr>
<td>10 ng</td>
<td>0.33 ± 0.21</td>
<td>0.06 ± 0.04**</td>
</tr>
<tr>
<td>1 ng</td>
<td>0.02 ± 0.02</td>
<td>0.002 ± 0.001</td>
</tr>
<tr>
<td>DY380mCM(−) reporter strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 ng</td>
<td>0.096 ± 0.020</td>
<td>0.044 ± 0.030*</td>
</tr>
<tr>
<td>10 ng</td>
<td>0.047 ± 0.02</td>
<td>0.007 ± 0.004**</td>
</tr>
<tr>
<td>1 ng</td>
<td>0.001 ± 0.001</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

SSOs containing phosphothioate (PT) or phosphothioate and 3’-terminal deoxycytidine (PT+ddC) modifications were introduced into DY380 cells containing episomal copies of the pGKfrt(CM+)−/+(−) reporter plasmids, or into the DY380mCM(+)−/+(−) reporter strains, to restore the function of a defective chloramphenicol resistance gene (mCM). Correction efficiencies were calculated from the relative number of transformant cells that were resistant to chloramphenicol. (+) and (−) indicate the orientation of the reporter gene relative to the direction of replication (Figure 1). *P < 0.05, **P < 0.01 versus correction efficiency using PT SSO.

Nonetheless, our results clearly demonstrate that the extension of the correction-SSO is important for efficient gene repair in both plasmid and chromosomal loci.

The majority of SSO-mediated gene repair events require origin-dependent DNA replication

To establish if there was a link between DNA replication and SSO-mediated gene repair, we constructed another reporter plasmid (p$m$Kan) that contained a temperature-sensitive pSC101 replication origin (30). Plasmid replication could be allowed to proceed normally by incubating the cells at 32°C, or suppressed by transferring the cells to temperatures of 37°C or higher. A number of controls were first performed to establish the reliability of the p$m$Kan reporter system, and to establish optimal experimental parameters. The inhibition of p$m$Kan replication was shown to be reversible, as demonstrated by the formation of similar numbers of colonies on selective LB-plates for two equivalent cultures of DY380/p$m$Kan that had either been incubated at 37°C for <90 min then transferred to 32°C, or had been incubated at 32°C continuously (data not shown). Furthermore, at 32°C, the efficiency of mutating the mKan gene on the p$m$Kan plasmid was essentially the same as when it was located on other plasmids (such as pmKan) that contained a wild-type ColE1 origin of replication (data not shown).

DNA replication was transiently suppressed by incubating the DY380/p$m$Kan cells at 37°C for various periods of time directly after transformation with the correction-SSO, followed by transferal to 32°C to allow replication to proceed (for a total of 120 min). It was found that when the cells were placed at 37°C for 60 min directly after electroporation with the SSO, the efficiency of SSO-mediated gene repair decreased by ∼85% (Figure 4, t = 60 min). This indicated that the inhibition of DNA replication during this period led to a substantial reduction in the efficiency of gene repair.

Mutations in the host replication machinery significantly alter SSO-mediated gene repair efficiencies

We then investigated whether various components of the host replication machinery were involved in the λ-Red/SSO-mediated gene repair process. To achieve this, we deleted or mutated a number of genes whose products are involved in replication, within the DY380mCM(+) and DY380mCM(−) chromosomal reporter strains. SSO-mediated gene repair assays analogous to those conducted above were performed in each strain, and efficiencies were compared.

A dnaQ null mutation increases gene repair efficiency by saturating host MMR machinery

In E.coli, different nucleotide mismatches are recognized and repaired by the MMR machinery with differing efficiencies: G/G mismatches are corrected the most efficiently, followed by A/G and T/C mismatches, with C/C mismatches poorly repaired (31). When equivalent SSOs encoding different central mismatches (i.e. G/G versus C/C) were used to correct the mCM reporter gene, we found differences in repair efficiencies that were consistent with the known mismatch-dependent specificities, and with results previously reported for λ-Red/SSO-mediated gene repair (20,32) [see Table 2].

The high levels of fidelity in DNA replication are dependent on the exonucleolytic proofreading activities of the dnaQ-encoded e subunit of the E.coli DNA polymerase III holoenzyme. A dnaQ null mutant was generated to examine how the absence of proofreading affected the putatively

Table 2. Saturation of MMR functions in a dnaQ strain results in elevated levels of gene repair

<table>
<thead>
<tr>
<th>Strain</th>
<th>% Correction efficiency using 89G oligo (leading strand, G/G mismatch)</th>
<th>% Correction efficiency using 89C oligo (lagging strand, C/C mismatch)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCM(+)</td>
<td>0.02 ± 0.01</td>
<td>1.425 ± 0.981</td>
</tr>
<tr>
<td>ΔmutS mCM(+)</td>
<td>0.12 ± 0.04*</td>
<td>1.786 ± 0.244</td>
</tr>
<tr>
<td>ΔdnaQ mCM(+)</td>
<td>0.40 ± 0.28*</td>
<td>0.714 ± 0.345</td>
</tr>
<tr>
<td>ΔmutSΔdnaQ mCM(+)</td>
<td>0.28 ± 0.18*</td>
<td>0.901 ± 0.395</td>
</tr>
</tbody>
</table>

Efficiencies of SSO-mediated gene repair were determined in DY380mCM reporter strains devoid of the proofreading activity of the E.coli polymerase III holoenzyme (ΔdnaQ) and/or MMR (ΔmutSΔdnaQ). (+) and (−) indicate the orientation of the reporter gene relative to the direction of replication, and ‘G/G’ and ‘C/C’ denote the mismatch imposed by the SSOs upon annealing to its homologous target. *P < 0.05 versus control.

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In E.coli, different nucleotide mismatches are recognized and repaired by the MMR machinery with differing efficiencies: G/G mismatches are corrected the most efficiently, followed by A/G and T/C mismatches, with C/C mismatches poorly repaired (31). When equivalent SSOs encoding different central mismatches (i.e. G/G versus C/C) were used to correct the mCM reporter gene, we found differences in repair efficiencies that were consistent with the known mismatch-dependent specificities, and with results previously reported for λ-Red/SSO-mediated gene repair (20,32) [see Table 2].

The high levels of fidelity in DNA replication are dependent on the exonucleolytic proofreading activities of the dnaQ-encoded e subunit of the E.coli DNA polymerase III holoenzyme. A dnaQ null mutant was generated to examine how the absence of proofreading affected the putatively
Suppression of DNA replication results in lower SSO-mediated gene repair efficiencies. In plasmids containing the pSC101 temperature-sensitive replication origin (p+mKan), the gene repair efficiencies are reduced if there is prolonged incubation at 37°C after electroporation with the correction-SSO. Competent DY380/p+mKan (shaded bars) and DY380/ pnKan (white bars) cells were electroporated with SSO, incubated at 37°C for various time periods to suppress DNA replication, before being transferred to a replication permitting temperature (32°C), for a total of 2 h post-electroporation. Aliquots were plated onto LB-agar containing ampicillin and LB-agar containing ampicillin + kanamycin, and incubated overnight at 32°C. Colonies were counted to determine the gene repair efficiencies.

Figure 4. Suppression of DNA replication results in lower SSO-mediated gene repair efficiencies. In plasmids containing the pSC101 temperature-sensitive replication origin (p+mKan), the gene repair efficiencies are reduced if there is prolonged incubation at 37°C after electroporation with the correction-SSO. Competent DY380/p+mKan (shaded bars) and DY380/ pnKan (white bars) cells were electroporated with SSO, incubated at 37°C for various time periods to suppress DNA replication, before being transferred to a replication permitting temperature (32°C), for a total of 2 h post-electroporation. Aliquots were plated onto LB-agar containing ampicillin and LB-agar containing ampicillin + kanamycin, and incubated overnight at 32°C. Colonies were counted to determine the gene repair efficiencies.

Figure 4. Suppression of DNA replication results in lower SSO-mediated gene repair efficiencies. In plasmids containing the pSC101 temperature-sensitive replication origin (p+mKan), the gene repair efficiencies are reduced if there is prolonged incubation at 37°C after electroporation with the correction-SSO. Competent DY380/p+mKan (shaded bars) and DY380/ pnKan (white bars) cells were electroporated with SSO, incubated at 37°C for various time periods to suppress DNA replication, before being transferred to a replication permitting temperature (32°C), for a total of 2 h post-electroporation. Aliquots were plated onto LB-agar containing ampicillin and LB-agar containing ampicillin + kanamycin, and incubated overnight at 32°C. Colonies were counted to determine the gene repair efficiencies.

Intriguingly, there was a 10-fold reduction in the gene repair efficiencies in the MMR defective strain (i.e. mutS) when compared with the wild-type strain (Table 2). This pattern was similar to those found in MMR defective strains i.e. ΔmutS [see ΔmutS(+)] and ΔmutS(−) mutants in Table 2, and in experiments described previously (20) and may be accounted for by differences in the repair efficiencies for the specific mismatches imposed by the SSOs (i.e. G/G versus C/C). As it has previously been reported that a lack of proofreading activity in the DNA polymerase III holoenzyme (ΔdnaQ) significantly disrupts its ability to associate with the DNA polymerase χy subunits (38,39). From hereon, we will collectively refer to these mutants as Δβ-clamp loading mutants.

We first introduced SSO 89G into DY380mCM(+) and the mutant reporter strains derived from it. As a leading strand correction-SSO, gene repair efficiencies were significantly enhanced in the sbb113ΔmCM(+), ΔholCmCM(+) and ΔholDmCM(+) strains when compared to the mCM(+) control. In contrast, when SSO 89C was used (which corresponds to the lagging strand), similar gene repair frequencies were obtained in the wild-type and Δβ-clamp loading mutant strains (Table 4). These selective increases in gene repair frequencies were similar to those found in the ΔdnaQmCM(+) and ΔmutSΔmCM(+) strains, where enhancements in gene repair efficiencies were observed for the G/G mismatch-imposing SSO 89G, but not the C/C mismatch-imposing SSO 89C (Tables 2 and 4). To test for mismatch-specific effects, we also constructed β-clamp loading mutants in DY380mCM(−), where the reporter allele is orientated in the opposite direction relative to that in DY380mCM(+) (Figure 1). Correction-SSOs 89G and 89C, which correspond to the lagging and leading strands, respectively, were similarly introduced to repair the mCM gene. In the ΔholCmCM(−) and sbb113mCM(−) reporter strains, SSO 89C repaired the CM gene with consistently elevated efficiencies when compared with DY380mCM(−), whereas when SSO 89G was used, there were essentially no differences in the numbers of gene repair events detected.

| Table 3. Deletion of large unpaired mismatches is not affected in dnaQ and mutS mutants |
|----------------------------------|----------------|
| Reporter strain                  | % CM' recombinants* |
|                                  | ΔmutS | ΔmutQ |
| DY380mCM(+)→bla                 | 0.16 ± 0.07 | 0.11 ± 0.09 | 0.23 ± 0.17 |
| DY380mCM(+)→loxP                 | 0.17 ± 0.06 | 0.22 ± 0.13 | 0.13 ± 0.06 |

The effects of inactivating MMR (ΔmutS) and the proofreading activity of the E.coli polymerase III holoenzyme (ΔdnaQ) on the efficiency of SSO-mediated gene deletion were investigated in reporter strains carrying 2 kb (DY380mCM(+)→bla), or 100 bp (DY380mCM(+)→loxP) insertions in the mCM gene sequence. Correction-SSO 89C was used to specifically remove these intervening DNA sequences, and restore CM resistance (89C sequence corresponds to the nascent lagging strand in replication).

β-clamp loading mutants exhibit higher frequencies of gene repair

To investigate whether reducing the processivity of DNA replication affected the efficiency of SSO-mediated gene repair, holC and holD deletion mutants were generated. The HolC and HolD proteins have been reported to facilitate the loading of the β-clamp onto the RNA primers during lagging strand synthesis (35,36). Similarly, we also tested a single-stranded DNA binding protein (ssb) point mutant: sbb113, whose specific effects on DNA replication have been well characterized (37). In sbb113, there is a single amino acid substitution encoded in the conserved C-terminal domain, significantly disrupting its ability to associate with the DNA polymerase χy subunits (38,39). From hereon, we will collectively refer to these mutants as β-clamp loading mutants.

We first introduced SSO 89G into DY380mCM(+) and the mutant reporter strains derived from it. As a leading strand correction-SSO, gene repair efficiencies were significantly enhanced in the sbb113ΔmCM(+), ΔholCmCM(+) and ΔholDmCM(+) strains when compared to the mCM(+) control. In contrast, when SSO 89C was used (which corresponds to the lagging strand), similar gene repair frequencies were obtained in the wild-type and β-clamp loading mutant strains (Table 4). These selective increases in gene repair frequencies were similar to those found in the ΔdnaQmCM(+) and ΔmutSΔmCM(+) strains, where enhancements in gene repair efficiencies were observed for the G/G mismatch-imposing SSO 89G, but not the C/C mismatch-imposing SSO 89C (Tables 2 and 4). To test for mismatch-specific effects, we also constructed β-clamp loading mutants in DY380mCM(−), where the reporter allele is orientated in the opposite direction relative to that in DY380mCM(+) (Figure 1).
E. coli, important for processivity of DNA replication in SS0-mediated gene repair efficiencies were determined for a number of ssb113DmCM(+) strains. Oligos 89G and 89C specifically correct the point-mutated mCM reporter gene, to restore chloramphenicol resistance. (+) and (-) indicate the orientation of the reporter gene relative to the direction of replication. ‘G/G’ and ‘C/C’ denote the mismatch imposed by the SS0s upon annealing to its homologous target. ND, not determined; *P < 0.05 versus control.

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<th>Table 4. Relationship between DNA replication processivity and efficiency of SS0-mediated gene repair in DY380mCM chromosomal reporter strains</th>
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<tr>
<td>DY380 Reporter strain</td>
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<td>-----------------------</td>
</tr>
<tr>
<td>mCM(+)</td>
</tr>
<tr>
<td>ΔholC mCM(+)</td>
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<tr>
<td>ΔholD mCM(+)</td>
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<td>ssb113mCM(+)</td>
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<tr>
<td>% Correction efficiency using 89C oligo (lagging strand, C/C mismatch)</td>
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<tr>
<td>-----------------------</td>
</tr>
<tr>
<td>mCM(−)</td>
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<tr>
<td>ΔholC mCM(−)</td>
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<td>ssb113mCM(−)</td>
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SS0-mediated gene repair efficiencies were determined for a number of chromosomal reporter strains containing mutations in various components important for processivity of DNA replication in E. coli. Oligos 89G and 89C specifically correct the point-mutated mCM reporter gene, to restore chloramphenicol resistance. (+) and (−) indicate the orientation of the reporter gene relative to the direction of replication. ‘G/G’ and ‘C/C’ denote the mismatch imposed by the SS0s upon annealing to its homologous target. ND, not determined; *P < 0.05, *P < 0.01 versus control.

Importantly, the increase in gene repair efficiencies associated with the use of the leading strand SS0 (89G) was much lower in the DY380mCM(−) derived β-clamp loading mutants, than in the corresponding DY380mCM(+) derivatives. This suggested that other factors, in addition to the nature of the SS0-encoded mismatch, were contributing to the elevated gene repair levels in the β-clamp loading mutants. In combination with the results from the ΔdnaQ mutant reporter strains, this further implicates the involvement of replication in SS0-directed gene repair.

Host recombination proteins are not required for SS0-catalyzed gene repair events

According to the ‘annealing-integration’ model for SS0-mediated gene repair, the SS0 is annealed and incorporated as a nascent strand at the replication fork. Hence it is possible that this phase protein-dependent process does not require endogenous recombination proteins. Although the DY380 strain is recA+, in order to investigate the potential involvement of other host recombination factors in the SS0-mediated repair of point mutations, we generated various mutant derivatives of the DY380mCM(+) reporter strains. As shown in Table 5, we observed no significant differences in gene repair efficiencies in mutants that carried null alleles of recF, recG, recQ or ruvC. These proteins play a number of roles in the formation and processing of various recombination intermediates in E. coli. Similarly, the inactivation of priA in the reporter strain had little effect on the efficiency of SS0-mediated repair of the point-mutated CM reporter gene. Intriguingly, the priC mutant exhibited a modest increase in gene repair frequencies when SS0 89G, but not SS0 89C, was used to restore the tyrosine codon. Taken together, these results suggest that λ-Red/SS0-mediated gene repair operates via a pathway that does not require host-encoded factors that are important for conventional homologous recombination within bacteria.

The SS0-independent formation of CM (+) colonies within the various mutant reporter strains was also examined. Although a number of the mutant strains, i.e. dnaQ, mutS and their respective double mutants, exhibited elevated frequencies of spontaneous reversion (Supplementary Material Table 8), this contribution was insignificant when compared to the numbers obtained for the SS0-mediated events (<0.002%)

In addition, we found that a number of the mutants generated for this study displayed compromised cell viabilities, as determined by colony forming assays (with samples removed from cultures during the exponential phase, Supplementary Material Table 8). However, survival rates for each strain following electroporation were similar (data not shown), ruling out any potential bias caused by compromised cell viability during delivery of the correction-SSOs.

DISCUSSION

In this study, we tested the validity of a proposed ‘annealing and replication-dependent integration’ model for λ-Red/SS0-mediated gene repair in E. coli, by systematically examining several aspects of the gene repair process.

We first investigated whether an SS0 was directly incorporated into its homologous dsDNA target during a gene repair event, by determining if either of the two internal biotin tags from a biotinylated correction-SS0 were retained in the successfully corrected plasmid product. We reasoned that the probability of a biotinylated dT residue, generated by intracellular核酸 digestion of the labeled SS0, being re-incorporated into the plasmid was negligible. Consequently, any biotinylated plasmid detected must have resulted from the direct incorporation of the correction-SSO into its target locus. As the actual amounts of corrected and biotinylated plasmid formed were extremely small, it was necessary to amplify the section of the plasmid that had been repaired (i.e. the kan gene), in order to verify the presence of the

Table 5. SS0-mediated gene repair efficiencies in DY380mCM(+) and a number of isogenic mutant reporter strains

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<tr>
<td>% CM (+) recombinants</td>
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</tr>
<tr>
<td>DY380mCM(+)</td>
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<td>ΔholC ΔrecFmCM(+)</td>
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label. This meant that a highly stringent washing protocol was required to remove any contaminating DNA species (especially pmKan DNA) that may have adsorbed non-specifically onto the streptavidin resin. Our successful detection of repaired biotinylated plasmid demonstrates for the first time that the correction-SSO is indeed physically incorporated into its dsDNA target during a λ-Red-mediated gene repair process.

Following the annealing event, the SSO is predicted to prime, or be incorporated into the nascent strand by the replication machinery. Consistent with this idea, we found that when a non-extendable SSO containing a chain-terminating 3’-dideoxycytidine (ddC) residue was used to repair a point mutation, fewer recombinants were formed than when the equivalent extendable SSO (containing a 3’-dC residue) was used. The requirement for extension of the correction-SSO suggests that it most probably anneals to a complementary region of single-stranded DNA at the target locus, prior to serving as a primer for DNA synthesis.

However, when this non-extendable SSO (PT+ddC SSO) was used, a residual level of gene repair remained. A trivial explanation is that the PT-modified termini are insufficient to protect the SSO from internal cleavage by endogenous endonucleases, hence DNA synthesis (extension) can proceed as proposed in the current model. Another possibility is that these residual gene repair events do not require the SSO to serve as a primer for DNA synthesis. It is possible that DNA replication reinitiates downstream of the annealed SSO, leaving a daughter strand gap that remains unrepaired until the next round of replication (40). It has also been proposed that a D-loop structure is formed when a correction-SSO invades and anneals with its target dsDNA sequence. Endonucleolytic cleavage of the resultant recombination intermediate allows subsequent ligation and assimilation of the SSO into the duplex DNA, resulting in a gene repair event (3). From the results of this one experiment alone, it is not possible to determine whether the gene correction events result from annealing and DNA polymerization (SSO extension) at the replication fork, or from SSO D-loop formation and processing. However, considering the fact that efficient gene repair also requires origin-dependent DNA replication (see below), we favor the former possibility, where the majority of SSO-mediated gene repair events occurs via extension of the correction-SSO at the replication fork.

Of notable interest was the variation in gene repair efficiencies when different amounts of PT+ddC SSO were electroporated into the cells. Specifically, the relative frequencies of gene repair were greatly reduced when lower amounts of PT+ddC SSO were used (see Table 1, e.g. 10 ng compared with 100 ng). We attributed this to small quantities of heterogeneous SSO species present in the commercially synthesized SSO preparation used. Consequently, when larger amounts of the SSO are used, there is a greater likelihood that the ‘impurities’ present, namely extendable SSOs that anneal to their specific target, to mediate the repair of the reporter gene.

The results from our transient inhibition experiments indicate that origin-dependent DNA replication plays a pivotal role in SSO-mediated gene repair. DNA replication provides transient regions of single-stranded DNA, which the correction-SSO may access. As the activities of the λ-Red proteins in DY380 are extremely limited after incubation at 37°C for 60 min, and are unlikely to support significant levels of gene repair activity (data not shown), we suspect that other cellular processes such as transcription might provide the required regions of single-stranded DNA when the replication of the target sequence is halted, accounting for the remaining 15% of gene repair events. Alternatively, this 15% may result from the incomplete suppression of pmKan DNA replication at 37°C. Another non-mutually exclusive possibility is that a fraction of these gene repair events arise via a mechanism other than the annealing-replication-integration pathway, perhaps by strand invasion of the Beta-SSO nucleoprotein filament (41) into its homologous dsDNA target (i.e. D-loop formation and subsequent processing).

When we consider that gene repair is more efficient when extendable SSOs are used, it appears likely that in addition to creating single-stranded regions, the host DNA replication machinery also promotes the incorporation of the SSO into the nascent strand after the annealing event. However, we cannot exclude other mechanisms by which DNA replication is involved in SSO-mediated gene repair. Interestingly, our observation that a residual level of gene repair activity remains when DNA replication is transiently inhibited, mirrors the results obtained with the non-extendable SSOs, suggesting that gene repair that occurs via alternative pathway(s) may not require extension of the SSO and/or DNA replication.

To further investigate the putative role played by the DNA replication machinery during SSO-mediated gene repair, we generated a number of mutants that were compromised in DNA replication fidelity and processivity. A lack of proof-reading activity in the DNA polymerase III holozyme led to significant increases in the levels of SSO-mediated gene repair. This effect is most likely due to a saturation of the host MMR machinery, as the efficiencies of SSO-mediated deletions of large heterologous sequences were not affected in either of the ΔmutQ or ΔmutS mutant reporter strains. More intriguingly, we observed notable increases in gene repair efficiencies in the β-clamp loading mutants: ΔholC, ΔholID and sshl113. These results indicate that the efficient loading of the DNA polymerase β-clamp plays an important role in the targeted gene repair process. A detailed investigation into the influence β-clamp loading events on the λ-Red/SSO-mediated recombination process is beyond the scope of the present work and is discussed elsewhere (M. S. Y. Huen et al., manuscript in preparation). Taken together, the enhanced levels of gene repair in the various constructed DNA replication mutant strains provide additional evidence supporting the formation of a replication intermediate during λ-Red/SSO-mediated gene repair in E.coli.

It has previously been reported that the efficiency of SSO-mediated gene repair is elevated in MMR defective mutants (20). Our results show that the ΔmutS and ΔmutQ mutants both exhibit similar patterns of increased gene repair efficiencies, dependent upon the nature of the mismatch encoded by the SSO. The E.coli MMR system utilizes the methylation status of dsDNA to differentiate the parental strand from the nascent strand in order to repair mismatches using the parental sequence as a template. Considering that newly synthesized DNA is rapidly re-methylated after replication
which are naturally out-competed by MutS, are able to act during a C/C mismatch repair event suggests the involvement of other mismatch repair factors present in the host MMR machinery, possibly due to the different DNA bending and unbending patterns upon recognition of this mismatch. The absence of the RecQ helicase and the RuvC resolvase, which normally participate in the resolution of recombination intermediates during homologous recombination in E. coli, had no influence on the frequency of SSO recombination events. The deletion of the PriA replication restart protein also had no significant effect on the efficiency of SSO-mediated gene repair. However, our finding that the deletion of the PriC primosome assembly protein led to a modest increase in the gene repair efficiency specifically when SSO 89G was used, warrants further investigation. It is possible that PriC normally participates in re-establishing the replication fork when the host replication machinery encounters a roadblock (44). We consider that both the PriA and PriC proteins play essential roles in re-assembling the replisome at D-loops and fork structures (44,45), and that priA mutants are defective in homologous recombination (46), it seems likely that the majority, if not all of the SSO-mediated gene repair events proceed via routes alternative to those used in conventional conjugation and transduction pathways.

To exclude the possibility of altered rates of clonal expansion in the various mutants after a gene repair event, we allowed the electroporated cells to recover for various lengths of time before plating (from 0 to 180 min post-electroporation). However, we observed no significant differences in the levels of recombinant formation over this 3 h period. This suggests that the gene repair efficiencies amongst the mutants are unlikely to be affected (at least during our experimental time frame) by a change in doubling time of the ‘corrected’ cells compared to the ‘uncorrected’ cells.

During the course of our experiments with the MMR defective strains, we found that when the leading strand SSO 89C was used to repair the mutant chloramphenicol resistance gene (mCM), the repair efficiency in the ΔmutS host was consistently lower than that found in the corresponding wild-type reporter strain (Table 2). However, this trend was not observed for the lagging strand SSO 89G. The C/C mismatch is known to be poorly repaired by the host MMR machinery, possibly due to the different DNA bending and unbending patterns upon recognition of this mismatch by the MutS protein (47). The next step was to determine if the ΔmutS strains displayed lowered repair efficiencies during a C/C mismatch repair event suggests the involvement of other mismatch repair systems. Indeed, the MutM and FabA proteins have been reported to bind to C/C mismatches in gel shift assays (48). Whether these and/or other repair systems, which are naturally out-competed by MutS, are able to act on these mismatch substrates in its absence awaits further examination. Similarly, competition between the MMR machinery and the MutY glycosylase for A/C mismpairs has been demonstrated in vivo (49).

The current model for λ-Red/SSO-mediated gene repair proposes that there is a single-stranded DNA annealing event at, or near the replication fork, followed by the integration of the SSO into a nascent strand. The putative ability of the λ-Red Beta protein to facilitate the direct annealing of SSOs to these transient regions of ssDNA eliminates the requirement for host-encoded recombination factors. When we consider that λ-Red/SSO-mediated gene repair is still highly efficient in the recA-deficient bacterial strains employed in this study, our results indicate the operation of a mechanism that is distinct from conventional homologous recombination between two dsDNA molecules: which is consistent with the single-strand annealing model.

Previous work has shown that SSOs can be successfully used to create specific, targeted mutations in yeast (50,51) and in mammalian cells (10). However the precise mechanism(s) by which SSOs mediate these genetic alterations remain to be fully established. Although organisms may utilize different sets of endogenous proteins to repair or alter DNA sequences, our results with an E. coli model suggest that the direct incorporation of correction-SSOs during the targeted gene repair process may occur in both bacteria and mammalian cells (12). Taken together with other experimental results supporting the involvement of replication in SSO-mediated gene repair in mammalian cells (10,52–54), our findings further strengthen the idea that SSO-mediated mutagenesis operates via a common mechanism across different species.

In summary, our results provide evidence strongly supporting the operation of an ‘annealing and replication-dependent integration’ model, in which the introduced correction-SSO is targeted to transiently single-stranded regions of homologous DNA at the replication fork. Our study indicates that there is a minimal requirement for host factors for efficient annealing of the SSO at one or more of the several replication forks present during the short life cycle of E. coli. Indeed, targeted repair of the point-mutated reporter gene was found to be independent of a number of host-encoded proteins that play important roles in DNA repair. We provide direct evidence showing that the majority of λ-Red/SSO-mediated gene repair events occur via single-stranded DNA annealing, and that the host replication machinery plays a key role in the mutagenic process. Our findings will help guide future experiments involving SSO-mediated gene repair in mammalian and prokaryotic cells, and suggest several mechanisms by which the efficiencies may be reliably and substantially increased.

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