

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

Michael S. Y. Huen<sup>1</sup>, Xin-tian Li<sup>1,2</sup>, Lin-Yu Lu<sup>1</sup>, Rory M. Watt<sup>3</sup>, De-Pei Liu<sup>2</sup>  
and Jian-Dong Huang<sup>1,\*</sup>

<sup>1</sup>Department of Biochemistry, The University of Hong Kong, 3/F Laboratory Block, Faculty of Medicine Building, 21 Sassoon Road, Pokfulam, Hong Kong SAR, China, <sup>2</sup>National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (CAMS) and Peking Union Medical College (PUMC), Beijing 100005, P.R. China and <sup>3</sup>Open Laboratory of Chemical Biology, The Institute of Molecular Technology for Drug Discovery and Synthesis, Department of Chemistry, The University of Hong Kong Pokfulam Road, Hong Kong SAR, China

Received May 25, 2006; Revised October 4, 2006; Accepted October 10, 2006

## 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  $\lambda$ -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 recombineering) 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  $\lambda$ -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.

\*To whom correspondence should be addressed. Tel: +852 2819 2810; Fax: +852 2855 1254; Email: jdhuang@hkucc.hku.hk

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- $\lambda$  Red recombination system (14–18). The efficiencies of both of 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  $\lambda$ -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  $\lambda$ -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, (25,26) 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  $\lambda$  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-mcrA* $\Delta$ (*mrr-hsdRMS-mcrBC*) $\Phi$ 80*dlacZ* $\Delta$  *M15*  $\Delta$ *lacX7 deoR recA1 endA1 ara* $\Delta$ 139 $\Delta$ (*ara, leu*)7649*galUgalKrspL-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  $\mu$ g ml<sup>-1</sup>, 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 *EagI* and *NcoI* digested 559 bp PCR fragment was purified then ligated into the 4287 bp *EagI*–*NcoI* fragment of PGK-*frt* to form pmKan.

**p<sup>ts</sup>mKan** pBluescript KS<sup>+</sup> (Stratagene) and pmKan were digested with *EcoRI* and *ClaI*. 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 *ClaI* and *SacI*. The 1813 and 6444 bp fragments were recovered from their respective plasmids and ligated to form p<sup>ts</sup>mKan.

### Verification of biotinylated-SSO incorporation

$\lambda$ -Red expression was induced in cultures of DY380/pmKan cells (in 50 ml LB medium in 250 ml Erlenmeyer flasks, grown to OD<sub>600</sub> 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  $\mu$ l) 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

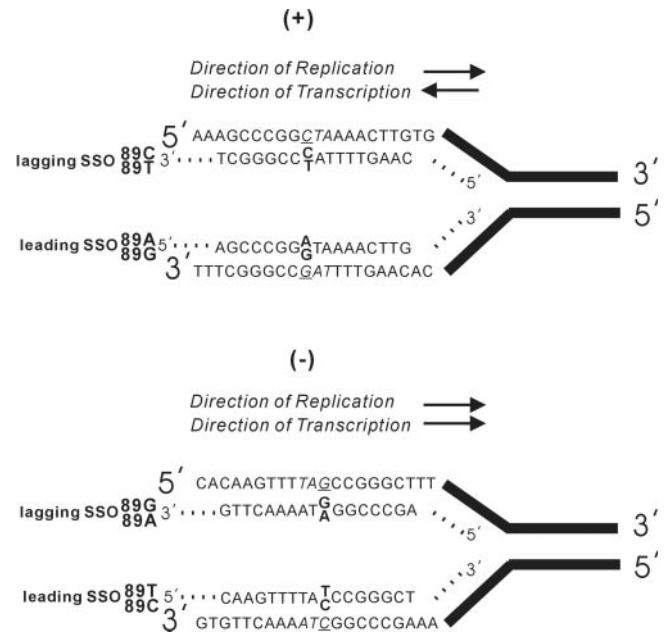
Overnight cultures of DY380/p<sup>ts</sup>mKan or DY380/pmKan were diluted 50 times in fresh LB medium, expanded until OD<sub>600</sub> = 0.6 at 32°C, then transferred to 42°C for 15 min to induce Red expression. The cells were chilled and subsequently electroporated with oligo 12A (100 ng) as described above, then LB media (1 ml) was added. Equal aliquots of the electroporated DY380/p<sup>ts</sup>mKan 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(+)<sub>F</sub> and mcm(-)<sub>F</sub> 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 *frt* sites was removed by transforming and inducing FLP expression carried on the 705-flp plasmid (Courtesy of Dr A. F. Stewart).

To generate the insertion mutant reporter DY380mCM(+)-bla (~2 kb insertion), a PCR-amplified β-lactamase cassette, which contained at its termini flanking regions homologous to the CAT gene either side of the point mutation, was recombined into DY380mCM(+). The β-lactamase gene in this cassette was sandwiched between two *loxP* site-specific recombination sequences (i.e. 'floxed'). This enabled its



**Figure 1.** Sequence of the point-mutated antibiotic resistance allele (mCM) and SSOs used to correct this mutation. Diagram shows the portion of the point-mutated chloramphenicol acetyltransferase gene (mCM) that contains the TAG amber (stop) codon. The single base mismatches imposed by the four different homologous correction oligos (SSO 89C, SSO 89G, SSO 89T, SSO 89A) to mutate this codon to tyrosine (and consequently restore normal gene function) are highlighted in bold. The reporter allele was inserted at the *bioA* locus, in both directions to create two strains: DY380CM(+/-), where (+) and (-) indicate the orientation of the mCM gene relative to the replication origin *oriC*. The directions of chromosomal replication and CM gene transcription in the (+) and (-) strains are indicated. Depending on the reporter strain used, the sequence of the correction-SSOs may correspond to either the replicative leading or lagging strands, and the transcriptional coding or template strands.

subsequent removal 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(+)<sub>loxP</sub>].

### Generating mutant derivatives

Using recombineering, 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



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<sup>-1</sup> 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<sup>r</sup>), 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<sup>r</sup> 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 OD<sub>600nm</sub> 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

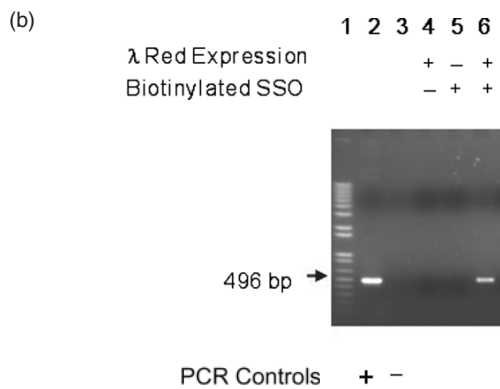
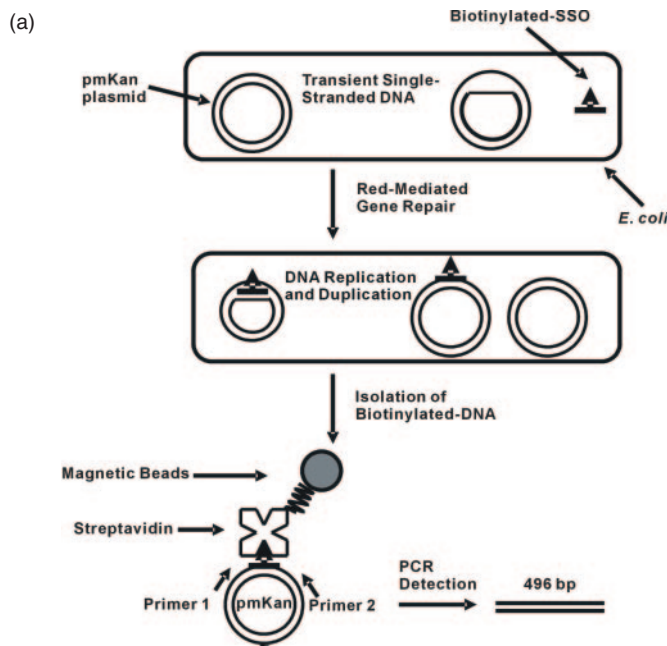
the *kan* gene meant that cells transformed with pmKan remained sensitive to kanamycin. A 91-mer correction-SSO (oligo 12A) was designed to convert the nonsense mutation (TAG) in the *kan* gene to a new silent mutation (TAC), restoring the gene function. The corrected pmKan DNA was isolated from individual 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

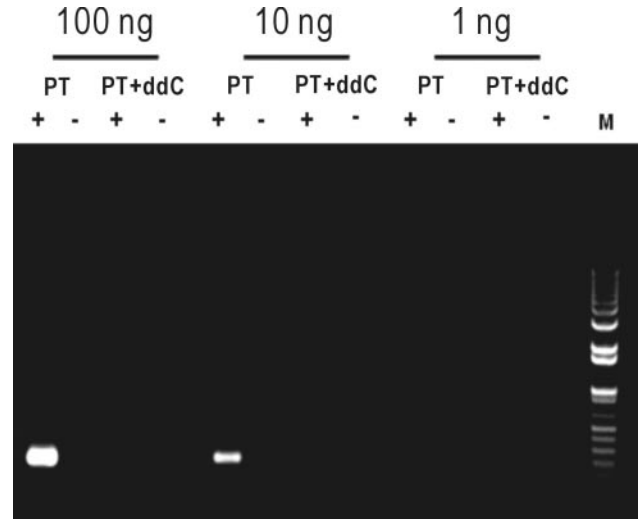
To test whether the correction-SSO served as a primer for DNA synthesis, we designed a 89mer that contained a central mismatch, with flanking sequences homologous to the point-mutant kanamycin reporter gene, as well as a chain-terminating 2',3'-dideoxycytidine (ddC) residue (PT+ddC SSO). The SSO was stabilized from exonuclease cleavage by incorporating PT linkages at both termini. An analogous SSO that contained similarly positioned PT linkages but a 3' terminal deoxycytidine residue (PT SSO), served as a control. We first confirmed in a PCR reaction (which included mCM(+)/DT2 as the reverse primer) that the dideoxy-terminating 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



**Figure 2.** Verification of SSO incorporation into its homologous DNA target (A) A schematic illustration of the experimental procedure. Biotinylated recombination products were purified using magnetic streptavidin beads. The presence of (corrected) pmKan was confirmed by the detection of a 496 bp PCR product. (B) pmKan and ddH<sub>2</sub>O were used as templates for the negative and positive PCR controls (lanes 2 and 3 respectively). DY380/pmKan cells were incubated at 42°C for 15 min to induce  $\lambda$ -Red protein expression prior to electroporation with biotinylated-SSO (lane 6) or unmodified SSO (lane 4). As a control, DY380/pmKan cells that had been incubated at 32°C for 15 min (i.e. no  $\lambda$ -Red induction) were similarly electroporated with biotinylated-SSO (lane 5). Plasmid DNA were extracted from the electroporated cells after a 15 min recovery period. Three independent experiments were performed; a representative experiment is shown.

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 -). The plasmid substrates were first established as stable episomes in DY380. Subsequently, the DY380/pGKfrtmCM cells were induced for  $\lambda$ -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).



**Figure 3.** Chain-terminating ddC residue prevents replicative extension *in vitro*. Primers containing 6 phosphothioate linkages at each terminus (PT SSO) or 6 phosphothioate linkages at each terminus and a 3'-dideoxycytidine residue (PT+ddC SSO) were used to amplify a 196 bp fragment using pGKfrtmCM(-) as template and mCM(+)/DT2 as the reverse primer, in a standard PCR reaction (see Table 6 in Supplementary material for primer sequences). PCRs were performed with the modified SSOs present at three different concentrations (1, 10 or 100 ng per reaction). Results show that the chain-terminating ddC nucleotide on the PT+ddC SSO is sufficient to prevent replicative extension by a DNA polymerase endowed with proofreading activity.

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(+) and DY380mCM(-). After induction of  $\lambda$ -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(-), 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

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

SSOs containing phosphothioate (PT) or phosphothioate and 3'-terminal dideoxycytidine (PT+ddC) modifications were introduced into DY380 cells containing episomal copies of the pGKfrtmCM(+)(-) 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<sup>ts</sup>mKan) 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<sup>ts</sup>mKan reporter system, and to establish optimal experimental parameters. The inhibition of p<sup>ts</sup>mKan 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<sup>ts</sup>mKan 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<sup>ts</sup>mKan 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<sup>ts</sup>mKan cells at 37°C for various periods of time directly after transformation with the correction-SSO, followed by transfer 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

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

DY380 Reporter Strain	% Correction efficiency using 89G oligo (leading strand, G/G mismatch)	% Correction efficiency using 89C oligo (lagging strand, C/C mismatch)
mCM(+)	0.02 ± 0.01	1.425 ± 0.981
$\Delta mutS$ mCM(+)	0.12 ± 0.04*	1.786 ± 0.244
$\Delta dnaQ$ mCM(+)	0.40 ± 0.28*	0.714 ± 0.345
$\Delta dnaQ \Delta mutS$ mCM(+)	0.28 ± 0.18*	0.901 ± 0.395
	% Correction efficiency using 89C oligo (leading strand, C/C mismatch)	% Correction efficiency using 89G oligo (lagging strand, G/G mismatch)
mCM(-)	0.17 ± 0.05	0.30 ± 0.02
$\Delta mutS$ mCM(-)	0.01 ± 0.01*	1.57 ± 1.16*
$\Delta dnaQ$ mCM(-)	0.14 ± 0.02	1.35 ± 0.35*
$\Delta dnaQ \Delta mutS$ mCM(-)	0.014 ± 0.007*	1.35 ± 0.82*

Efficiencies of SSO-mediated gene repair were determined in DY380mCM reporter strains devoid of the proofreading activity of the *E. coli* polymerase III holoenzyme ( $\Delta dnaQ$ ) and/or MMR ( $\Delta dnaQ \Delta mutS$ ). (+) 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.

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

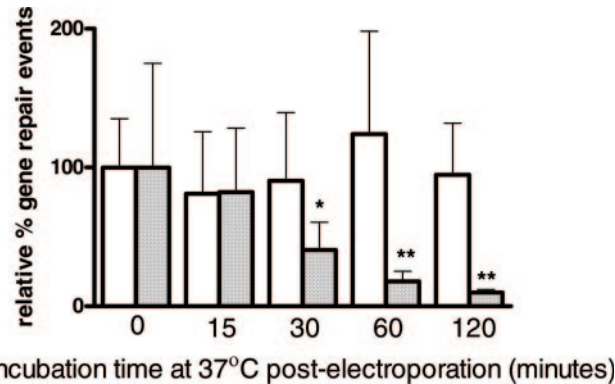
We then investigated whether various components of the host replication machinery were involved in the  $\lambda$ -Red/SSO-mediated gene repair process. To achieve this, we deleted or mutated a number of genes whose products are involved in replication, within the DY380mCM(+) and DY380mCM(-) chromosomal 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  $\lambda$ -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  $\epsilon$  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





**Figure 4.** Suppression of DNA replication results in lower SSO-mediated gene repair efficiencies. In plasmids containing the pSC101 temperature-sensitive replication origin ( $p^S$ mKan), the gene repair efficiencies are reduced if there is prolonged incubation at 37°C after electroporation with the correction-SSO. Competent DY380/ $p^S$ mKan (shaded bars) and DY380/pmKan (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. The y-axis shows the relative percentage of Kan<sup>r</sup> recombinant colonies that were formed for each time point, normalized to the number that were formed without incubation at 37°C post-electroporation (time 0). \* $P < 0.05$  and \*\* $P < 0.01$  versus time 0.

replication-dependent gene repair process. Gene repair efficiencies were greatly enhanced in the  $\Delta dnaQ$ mCM(+) reporter strain compared to the mCM(+) control when SSO 89G was used (Table 2). However, when the SSO 89C was used, there was little difference in the frequency of gene repair. This pattern was similar to those found in MMR defective strains i.e.  $\Delta mutS$  [see  $\Delta mutS(+)$  and  $\Delta mutS(-)$  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 saturates the MMR system (33), we tested double mutants that had been rendered defective in both replicative DNA proofreading and MMR activities i.e.  $\Delta dnaQ$  and  $\Delta mutS$ . We found no significant differences in targeted gene repair levels between the  $\Delta dnaQ$  and  $\Delta dnaQ\Delta mutS$  double mutant strains (Table 2).

Intriguingly, there was a 10-fold reduction in the gene repair levels in the MMR defective DY380mCM(-) reporter strain (i.e.  $\Delta mutS$ ), when the C/C mismatch-imposing SSO (i.e. 89C) was used to restore the antibiotic function. To exclude the possibility of suppressor or spontaneous mutation(s) in addition to the intended  $mutS$  deletion, we examined several independent DY380(-) $\Delta mutS$ mCM clones, and identical results were obtained. This phenomenon remains elusive and is further discussed below.

Based on the mismatch-dependent repair abilities of the *E. coli* MMR system, and the observation that it does not readily repair large unpaired heterologous sequences (34), we hypothesized that the deletion of *dnaQ* would have a negligible effect on the efficiency of  $\lambda$ -Red/SSO-mediated deletion of relatively large stretches of DNA. Consistent with this hypothesis, we did not detect any significant differ-

**Table 3.** Deletion of large unpaired mismatches is not affected in *dnaQ* and *mutS* mutants

Reporter strain	% CM <sup>r</sup> recombinants*		
	-	$\Delta mutS$	$\Delta dnaQ$
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 ( $\Delta mutS$ ) and the proofreading activity of the *E. coli* polymerase III holoenzyme ( $\Delta dnaQ$ ) 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).

ences in efficiency for SSO-mediated deletion of a 100 bp or 2 kb stretch of DNA in either the  $\Delta mutS$  or  $\Delta dnaQ$  strains (Table 3).

### **$\beta$ -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  $\beta$ -clamp onto the RNA primers during lagging strand synthesis (35,36). Similarly, we also tested a single-stranded DNA binding protein (*ssb*) point mutant: *ssb113*, whose specific effects on DNA replication have been well characterized (37). In *ssb113*, there is a single amino acid substitution encoded in the conserved C-terminal domain, significantly disrupting its ability to associate with the DNA polymerase  $\chi\psi$  subunits (38,39). From hereon, we will collectively refer to these mutants as  $\beta$ -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 *ssb113*mCM(+),  $\Delta holC$ mCM(+) and  $\Delta holD$ mCM(+) 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  $\beta$ -clamp loading mutant strains (Table 4). These selective increases in gene repair frequencies were similar to those found in the  $\Delta dnaQ$ mCM(+) and  $\Delta mutS$ mCM(+) 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  $\beta$ -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  $\Delta holC$ mCM(-) and *ssb113*mCM(-) 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 4.** Relationship between DNA replication processivity and efficiency of SSO-mediated gene repair in DY380mCM chromosomal reporter strains

DY380 Reporter strain	% Correction efficiency using 89G oligo (leading strand, G/G mismatch)	% Correction efficiency using 89C oligo (lagging strand, C/C mismatch)
mCM(+)	0.020 ± 0.01	1.43 ± 0.98
<i>ΔholC</i> mCM(+)	0.26 ± 0.23**	2.02 ± 1.71
<i>ΔholD</i> mCM(+)	0.28 ± 0.19**	1.35 ± 0.64
<i>ssb113</i> mCM(+)	0.20 ± 0.16**	1.72 ± 1.60
	% Correction efficiency using 89C oligo (leading strand, C/C mismatch)	% Correction efficiency using 89G oligo (lagging strand, G/G mismatch)
mCM(-)	0.17 ± 0.15	0.30 ± 0.09
<i>ΔholC</i> mCM(-)	0.37 ± 0.22*	0.42 ± 0.16*
<i>ssb113</i> mCM(-)	0.36 ± 0.17*	0.33 ± 0.17

SSO-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 SSOs 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 SSO (89G) was much lower in the DY380mCM(-) derived  $\beta$ -clamp loading mutants, than in the corresponding DY380mCM(+) derivatives. This suggested that other factors, in addition to the nature of the SSO-encoded mismatch, were contributing to the elevated gene repair levels in the  $\beta$ -clamp loading mutants. In combination with the results from the *ΔdnaQ* mutant reporter strains, this further implicates the involvement of replication in SSO-directed gene repair.

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

According to the 'annealing-integration' model for SSO-mediated gene repair, the SSO is annealed and incorporated as a nascent strand at the replication fork. Hence it is possible that this phage protein-dependent process does not require endogenous recombination proteins. Although the DY380 strain is *recA*<sup>-</sup>, in order to investigate the potential involvement of other host recombination factors in the SSO-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 SSO-mediated repair of the point-mutated CM reporter gene. Intriguingly, the *priC* mutant exhibited a modest increase in gene repair frequencies when SSO 89G, but not SSO 89C, was used to restore the tyrosine codon. Taken together, these results suggest that  $\lambda$ -Red/SSO-mediated gene repair operates via a pathway that does

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

	% CM <sup>r</sup> recombinants 89G oligo (leading strand, G/G mismatch)	89C oligo (lagging strand, C/C mismatch)
DY380mCM(+)	0.020 ± 0.013	1.43 ± 0.98
DY380 <i>ΔrecF</i> mCM(+)	0.019 ± 0.006	1.28 ± 1.00
DY380 <i>ΔrecG</i> mCM(+)	0.016 ± 0.015	1.67 ± 1.38
DY380 <i>ΔrecQ</i> mCM(+)	0.015 ± 0.002	0.82 ± 0.30
DY380 <i>ΔruvC</i> mCM(+)	0.016 ± 0.010	1.38 ± 1.09
D380 <i>ΔpriA</i> mCM(+)	0.011 ± 0.011	1.04 ± 0.81
DY380 <i>ΔpriC</i> mCM(+)	0.050 ± 0.027*	0.79 ± 0.49

The efficiencies of SSO-mediated repair of a point-mutated chloramphenicol reporter gene, as indicated by levels of CM<sup>r</sup> colony formation, were measured in wild-type DY380mCM(+), as well as a number of reporter strains carrying mutations in several key proteins involved in homologous recombination. Correction-SSOs 89G and 89C correspond to the nascent leading and lagging strands, respectively. 'G/G' and 'C/C' denote the mismatch imposed by the SSO upon annealing to its homologous target. \* $P < 0.05$  vs DY380mCM(+).

not require host-encoded factors that are important for conventional homologous recombination within bacteria.

The SSO-independent formation of CM<sup>r</sup> 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 SSO-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  $\lambda$ -Red/SSO-mediated gene repair in *E. coli*, by systematically examining several aspects of the gene repair process.

We first investigated whether an SSO 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-SSO were retained in the successfully corrected plasmid product. We reasoned that the probability of a biotinylated dT residue, generated by intracellular nuclease digestion of the labeled SSO, 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



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  $\lambda$ -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

$\lambda$ -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 p<sup>ts</sup>mKan 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 holoenzyme 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  $\Delta dnaQ$  or  $\Delta mutS$  mutant reporter strains. More intriguingly, we observed notable increases in gene repair efficiencies in the  $\beta$ -clamp loading mutants:  $\Delta holC$ ,  $\Delta holD$  and  $ssb113$ . These results indicate that the efficient loading of the DNA polymerase  $\beta$ -clamp plays an important role in the targeted gene repair process. A detailed investigation into the influence  $\beta$ -clamp loading exerts on the  $\lambda$ -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  $\lambda$ -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  $\Delta mutS$  and  $\Delta dnaQ$  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

(42), the fact that elevated levels of recombinant formation can be observed in MMR-deficient strains further supports the idea of an incorporation event at the replication fork.

Finally, we sought to establish some of the genetic requirements for efficient SSO-mediated gene repair in *E. coli*. Using a mutant antibiotic reporter system, we evaluated targeted gene repair efficiencies in various strains that carried null alleles of host factors important for homologous recombination (Table 5). In sharp contrast to the proposed role played by the RecG helicase during the  $\lambda$  Red-mediated recombination of dsDNA molecules (43), we did not observe any dependence on RecG when SSOs were used to direct the repair of a chromosomal reporter allele (Table 5). Similarly, none of the other host recombination factors tested in this study significantly affected the SSO-mediated repair efficiencies. 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). If we consider that both the PriA and PriC proteins play essential roles in reassembling 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  $\Delta mutS$  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 fact that these  $\Delta mutS$  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 mispairs has been demonstrated *in vivo* (49).

The current model for  $\lambda$ -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  $\lambda$ -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  $\lambda$ -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  $\lambda$ -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.

## ACKNOWLEDGEMENTS

We thank Drs Donald Court and Daiguan Yu for the *E. coli* DY380 strain; and Dr. Francis Stewart for the 705-cre, 705-flp, pGK-*frt* and pGK-*loxP* plasmids. M.S.Y.H. thanks Drs Shirley Sy, Neal Copeland and Nancy Jenkins for insightful discussion towards this work. The authors also thank Dr Julian Tanner for help in editing the manuscript. This work

was supported by a joint grant from the Research Grant Council of Hong Kong and the National Science Foundation to J.-D.H. and D.-P.L. Funding to pay the Open Access publication charges for this article was provided by RGC.

*Conflict of interest statement.* None declared.

## REFERENCES

- Richardson,P.D., Kren,B.T. and Steer,C.J. (2001) Targeted gene correction strategies. *Curr. Opin. Mol. Ther.*, **3**, 327–337.
- Copeland,N.G., Jenkins,N.A. and Court,D.L. (2001) Recombineering: a powerful new tool for mouse functional genomics. *Nature Rev. Genet.*, **2**, 769–779.
- Parekh-Olmedo,H., Ferrara,L., Brachman,E. and Kmiec,E.B. (2005) Gene therapy progress and prospects: targeted gene repair. *Gene Ther.*, **12**, 639–646.
- Liu,L., Parekh-Olmedo,H. and Kmiec,E.B. (2003) The development and regulation of gene repair. *Nature Rev. Genet.*, **4**, 679–689.
- Brachman,E.E. and Kmiec,E.B. (2002) The 'biased' evolution of targeted gene repair. *Curr. Opin. Mol. Ther.*, **4**, 171–176.
- Rice,M.C., Czymmek,K. and Kmiec,E.B. (2001) The potential of nucleic acid repair in functional genomics. *Nat. Biotechnol.*, **19**, 321–326.
- Kmiec,E.B. (2003) Targeted gene repair—in the arena. *J. Clin. Invest.*, **112**, 632–636.
- Sullenger,B.A. (2003) Targeted genetic repair: an emerging approach to genetic therapy. *J. Clin. Invest.*, **112**, 310–311.
- Ellis,H.M., Yu,D., DiTizio,T. and Court,D.L. (2001) High efficiency mutagenesis, repair, and engineering of chromosomal DNA using single-stranded oligonucleotides. *Proc. Natl Acad. Sci. USA*, **98**, 6742–6746.
- Wu,X.S., Xin,L., Yin,W.X., Shang,X.Y., Lu,L., Watt,R.M., Cheah,K.S., Huang,J.D., Liu,D.P. and Liang,C.C. (2005) Increased efficiency of oligonucleotide-mediated gene repair through slowing replication fork progression. *Proc. Natl Acad. Sci. USA*, **102**, 2508–2513.
- Ferrara,L. and Kmiec,E.B. (2004) Camptothecin enhances the frequency of oligonucleotide-directed gene repair in mammalian cells by inducing DNA damage and activating homologous recombination. *Nucleic Acids Res.*, **32**, 5239–5248.
- Radecke,S., Radecke,F., Peter,I. and Schwarz,K. (2006) Physical incorporation of a single-stranded oligodeoxynucleotide during targeted repair of a human chromosomal locus. *J. Gene. Med.*, **8**, 217–228.
- Court,D.L., Sawitzke,J.A. and Thomason,L.C. (2002) Genetic engineering using homologous recombination. *Annu. Rev. Genet.*, **36**, 361–388.
- Zhang,Y., Buchholz,F., Muyrers,J.P.P. and Stewart,A.F. (1998) A new logic for DNA engineering using recombination in *Escherichia coli*. *Nature Genet.*, **20**, 123–128.
- Yu,D., Ellis,H.M., Lee,E.C., Jenkins,N.A., Copeland,N.G. and Court,D.L. (2000) An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **97**, 5978–5983.
- Datsenko,K.A. and Wanner,B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl Acad. Sci. USA*, **97**, 6640–6645.
- Murphy,K.C. (1998) Use of bacteriophage lambda recombination functions to promote gene replacement in *Escherichia coli*. *J. Bacteriol.*, **180**, 2063–2071.
- Murphy,K.C., Campellone,K.G. and Poteete,A.R. (2000) PCR-mediated gene replacement in *Escherichia coli*. *Gene*, **246**, 321–330.
- Swaminathan,S., Ellis,H.M., Waters,L.S., Yu,D., Lee,E.C., Court,D.L. and Sharan,S.K. (2001) Rapid engineering of bacterial artificial chromosomes using oligonucleotides. *Genesis*, **29**, 14–21.
- Li,X.T., Costantino,N., Lu,L.Y., Liu,D.P., Watt,R.M., Cheah,K.S., Court,D.L. and Huang,J.D. (2003) Identification of factors influencing strand bias in oligonucleotide-mediated recombination in *Escherichia coli*. *Nucleic Acids Res.*, **31**, 6674–6687.
- Thaler,D.S., Stahl,M.M. and Stahl,F.W. (1987) Double-chain-cut sites are recombination hotspots in the Red pathway of phage lambda. *J. Mol. Biol.*, **195**, 75–87.
- Takahashi,N. and Kobayashi,I. (1990) Evidence for the double-strand break repair model of bacteriophage lambda recombination. *Proc. Natl Acad. Sci. USA*, **87**, 2790–2794.
- Muyrers,J.P., Zhang,Y., Buchholz,F. and Stewart,A.F. (2000) RecE/RecT and Red $\alpha$ /Red $\beta$  initiate double-stranded break repair by specifically interacting with their respective partners. *Genes Dev.*, **14**, 1971–1982.
- Little,J.W. (1967) An exonuclease induced by bacteriophage lambda. II. Nature. of the enzymatic reaction. *J. Biol. Chem.*, **242**, 679–686.
- Li,Z., Karakousis,G., Chiu,S.K., Reddy,G. and Radding,C.M. (1998) The beta protein of phage lambda promotes strand exchange. *J. Mol. Biol.*, **276**, 733–744.
- Kmiec,E. and Holloman,W.K. (1981) Beta protein of bacteriophage lambda promotes renaturation of DNA. *J. Biol. Chem.*, **256**, 12636–12639.
- Poteete,A.R. (2001) What makes the bacteriophage lambda Red system useful for genetic engineering: molecular mechanism and biological function. *FEMS Microbiol. Lett.*, **201**, 9–14.
- Edwards,J.R., Itagaki,Y. and Ju,J. (2001) DNA sequencing using biotinylated dideoxynucleotides and mass spectrometry. *Nucleic Acids Res.*, **29**, E104.
- Lee,E.C., Yu,D., Martinez de Velasco,J., Tessarollo,L., Swing,D.A., Court,D.L., Jenkins,N.A. and Copeland,N.G. (2001) A highly efficient *Escherichia coli*-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics*, **73**, 56–65.
- Hashimoto-Gotoh,T. and Sekiguchi,M. (1977) Mutations of temperature sensitivity in R plasmid pSC101. *J. Bacteriol.*, **131**, 405–412.
- Su,S.S., Lahue,R.S., Au,K.G. and Modrich,P. (1988) Mismatch specificity of methyl-directed DNA mismatch correction in vitro. *J. Biol. Chem.*, **263**, 6829–6835.
- Costantino,N. and Court,D.L. (2003) Enhanced levels of lambda Red-mediated recombinants in mismatch repair mutants. *Proc. Natl Acad. Sci. USA*, **100**, 15748–15753.
- Fijalkowska,I.J. and Schaaper,R.M. (1995) Effects of *Escherichia coli* dnaE mutator alleles in a proofreading-deficient mutD5 strain. *J. Bacteriol.*, **177**, 5979–5986.
- Parker,B.O. and Marinus,M.G. (1992) Repair of DNA heteroduplexes containing small heterologous sequences in *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **89**, 1730–1734.
- Olson,M.W., Dallmann,H.G. and McHenry,C.S. (1995) DnaX complex of *Escherichia coli* DNA polymerase III holoenzyme. The chi psi complex functions by increasing the affinity of tau and gamma for delta.deltai' to a physiologically relevant range. *J. Biol. Chem.*, **270**, 29570–29577.
- Xiao,H., Crombie,R., Dong,Z., Onrust,R. and O'Donnell,M. (1993) DNA polymerase III accessory proteins. III. holC and holD encoding chi and psi. *J. Biol. Chem.*, **268**, 11773–11778.
- Chase,J.W., L'Italien,J.J., Murphy,J.B., Spicer,E.K. and Williams,K.R. (1984) Characterization of the *Escherichia coli* SSB-113 mutant single-stranded DNA-binding protein. Cloning of the gene, DNA and protein sequence analysis, high pressure liquid chromatography peptide mapping, and DNA-binding studies. *J. Biol. Chem.*, **259**, 805–814.
- Witte,G., Urbanke,C. and Curth,U. (2003) DNA polymerase III chi subunit ties single-stranded DNA binding protein to the bacterial replication machinery. *Nucleic Acids Res.*, **31**, 4434–4440.
- Glover,B.P. and McHenry,C.S. (1998) The chi psi subunits of DNA polymerase III holoenzyme bind to single-stranded DNA-binding protein (SSB) and facilitate replication of an SSB-coated template. *J. Biol. Chem.*, **273**, 23476–23484.
- Heller,R.C. and Marians,K.J. (2006) Replication fork reactivation downstream of a blocked nascent leading strand. *Nature*, **439**, 557–562.
- Rybalchenko,N., Golub,E.I., Bi,B. and Radding,C.M. (2004) Strand invasion promoted by recombination protein beta of coliphage lambda. *Proc. Natl Acad. Sci. USA*, **101**, 17056–17060.
- Stancheva,I., Koller,T. and Sogo,J.M. (1999) Asymmetry of Dam methylation on the leading and lagging arms of plasmid replicative intermediates. *EMBO J.*, **18**, 6542–6551.



43. Poteete, A.R., Fenton, A.C. and Murphy, K.C. (1999) Roles of RuvC and RecG in phage lambda red-mediated recombination. *J. Bacteriol.*, **181**, 5402–5408.
44. Heller, R.C. and Marians, K.J. (2005) The disposition of nascent strands at stalled replication forks dictates the pathway of replisome loading during restart. *Mol. Cell.*, **17**, 733–743.
45. Michel, B., Grompone, G., Flores, M.J. and Bidnenko, V. (2004) Multiple pathways process stalled replication forks. *Proc. Natl Acad. Sci. USA*, **101**, 12783–12788.
46. Kogoma, T., Cadwell, G.W., Barnard, K.G. and Asai, T. (1996) The DNA replication priming protein, PriA, is required for homologous recombination and double-strand break repair. *J. Bacteriol.*, **178**, 1258–1264.
47. Wang, H., Yang, Y., Schofield, M.J., Du, C., Fridman, Y., Lee, S.D., Larson, E.D., Drummond, J.T., Alani, E., Hsieh, P. *et al.* (2003) DNA bending and unbending by MutS govern mismatch recognition and specificity. *Proc. Natl Acad. Sci. USA*, **100**, 14822–14827.
48. Nakahara, T., Zhang, Q.M., Hashiguchi, K. and Yonei, S. (2000) Identification of proteins of *Escherichia coli* and *Saccharomyces cerevisiae* that specifically bind to C/C mismatches in DNA. *Nucleic Acids Res.*, **28**, 2551–2556.
49. Kim, M., Huang, T. and Miller, J.H. (2003) Competition between MutY and mismatch repair at A x C mispairs *In vivo*. *J. Bacteriol.*, **185**, 4626–4629.
50. Moerschell, R.P., Tsunasawa, S. and Sherman, F. (1988) Transformation of yeast with synthetic oligonucleotides. *Proc. Natl Acad. Sci. USA*, **85**, 524–528.
51. Liu, L., Rice, M.C., Drury, M., Cheng, S., Gamper, H. and Kmiec, E.B. (2002) Strand bias in targeted gene repair is influenced by transcriptional activity. *Mol. Cell. Biol.*, **22**, 3852–3863.
52. Brachman, E.E. and Kmiec, E.B. (2004) DNA replication and transcription direct a DNA strand bias in the process of targeted gene repair in mammalian cells. *J. Cell. Sci.*, **117**, 3867–3874.
53. Ferrara, L., Parekh-Olmedo, H. and Kmiec, E.B. (2004) Enhanced oligonucleotide-directed gene targeting in mammalian cells following treatment with DNA damaging agents. *Exp. Cell. Res.*, **300**, 170–179.
54. Brachman, E.E. and Kmiec, E.B. (2005) Gene repair in mammalian cells is stimulated by the elongation of S phase and transient stalling of replication forks. *DNA Repair (Amst)*, **4**, 445–457.