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Active transcription promotes single-stranded oligonucleotide mediated gene repair

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Abstract

The λ-Red-mediated recombination has been exploited as an efficient means for DNA manipulation. We previously reported that replication plays a pivotal role during this process. Replication direction dictates strand bias, such that single stranded oligonucleotide (SSO) with sequence corresponding to the nascent lagging strand directs higher levels of recombinant formation compared to its complementary SSO. In addition, the *Escherichia coli* methyl-directed mismatch repair system impedes efficient SSO-mediated site-specific gene repair. However, the role of transcription in determining strand bias and recombination efficiency is unclear. To address the potential role of transcriptional processes, we constructed plasmid substrates that harbor a mutant antibiotic reporter under the control of an inducible promoter. We found that transcription activation can promote recombinant formation to more than 10-folds whilst it has negligible effect on strand bias. Our findings provide evidence for a role of transcription in SSO-mediated gene repair process.

Keywords: λ-Red; Recombination; Strand bias; Gene therapy

The λ-Red recombination system has served as a powerful methodology for DNA modification [1–3]. Synthetic single-stranded oligonucleotides (SSOs) can be used efficiently as substrates to generate subtle mutations on either episomal or chromosomal loci. Unlike conventional cloning strategy, the phage-encoded recombination function has no requirement for restriction sites, and can utilize SSOs of relative short homology (~30 nt) for efficient manipulation of DNA sequences. Despite its numerous applications, the mechanistic basis for the λ-Red/SSO-mediated recombination remains to be explored.

Replication plays a pivotal role during λ-Red-mediated recombination between a SSO and its double-strand DNA target [4]. The current "annealing–integration" model proposes that the mutagenic SSO is annealed, by the phage-encoded single-strand annealing protein β, to its target at the replication fork. The host replication machinery subsequently incorporates the SSO as a nascent strand during DNA synthesis. One prevalent evidence for this model comes from the observation that the direction of replication dictates the strand bias effect, such that the SSO corresponding in sequence to the nascent lagging strand always directs higher level of recombination than its complementary sequence [5,6]. This has been attributed to the relative single-strandedness of the lagging strand template, which renders the nucleoprotein filament increased accessibility to its target DNA sequence.

Moreover, methyl-directed mismatch repair deficient (MMR) cells displayed elevated levels of recombination

Abbreviations: SSO(s), single-strand oligonucleotide(s); MMR, methyl-directed mismatch repair; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; oligo(s), oligonucleotide(s).

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frequencies [5,7]. The *Escherichia coli* MMR system utilizes the methylation status, namely the adenine methylation at the sequence GATC, of the newly replicated DNA to repair mismatches using the parental strand as template [8]. Since the half-life of hemi-methylation behind the replication fork corresponds to a few seconds [9], the transient nature of such hemi-methylated DNA allows post-replicative repair of potential mismatches that have escaped the proofreading activity endowed by the host DNA polymerase III holoenzyme. Considering the innate characteristics of the *E. coli* MMR system, that MMR deficient cells are more recombinogenic provides additional support for the notion that λ-Red/SSO operates via an annealing event at the replication fork.

In contrast to the prominent role of replication during λ-Red/SSO-mediated recombination in *E. coli*, accumulating evidence suggests that transcription plays a more important role in SSO-mediated gene repair in different mammalian models. Strand bias is an universal phenomenon during SSO-mediated gene repair in both prokaryotes and eukaryotes, and that in most cases, the direction of transcription has been shown to favor SSOs that correspond in sequence to the transcribed strand during targeted repair processes in mammalian systems [10–12].

The apparent dissimilarity in strand bias determination across the models prompted us to investigate whether transcriptional processes might also be involved in λ-Red/SSO-mediated recombinant formation in bacteria. Here, we report our studies on how DNA replication and transcription might affect SSO-mediated gene repair in *E. coli*.

Materials and methods

Oligonucleotides. Oligonucleotides were purchased in a desalted form without further purification (Pro-oligo, Singapore). Sequences of oligonucleotides are listed in Supplementary Table 4.

Genotype of strains. All strains used in this study are derived from DY380 and DY330 [3] and listed in Supplementary Table 5. Methods for generating the various reporter strains and their mutant derivatives are described in our previous publications [4,14,15].

Recombination assays using a mutant antibiotic reporter system. DY380 and derivatives were grown and induced essentially as described [13]. Briefly, single colony was inoculated and grown at 32 °C overnight then diluted 50-folds in LB media and allowed to expand until reaching an $OD_{600\ nm}$ of 0.4. λ-Red functions were induced by shaking the cultures in a 42 °C water bath for 15 min. Cells were quickly chilled on ice and washed with ice-cold ddH^2O for three times. The pellet was resuspended into 200 μl ddH^2O. A 50 μl aliquot of recombinogenic bacterial cells was used for each transformation. 100 ng of SSO was electroporated 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 chloramphenicol. Recombination efficiency was calculated by dividing the number of colonies that formed after 24 h on antibiotic plates and plain LB agar plates.

For assays conducted using the inducible plasmid reporters, overnight cultures of bacterial cells harboring the mCAT plasmids were expanded as above for 1 h and subsequently incubated in the presence of 0.4% glucose or 0.4% arabinose to repress or induce expression of the mutant reporters, respectively. λ-Red expression was induced and the cells were made electrocompetent. After electroporation of correction-SSO, transformed cells were incubated in fresh LB medium containing either glucose or arabinose for different lengths of time prior to plating onto LB agar supplemented with 0.4% arabinose.

Results

We and others previously reported that the direction of replication determines strand bias during λ-Red/SSO-mediated recombination in *E. coli* [5,6]. To consolidate this finding and to address the potential roles of transcription, we constructed mutant antibiotic reporters that result in expression of either a non-functional truncated chloramphenicol acetyltransferase (mCAT) or a kanamycin resistance gene (mKan) product. For the mCAT reporter, an amber mutation was generated to replace a tyrosine codon in its coding sequence. The mutant CAT allele was integrated in both orientations relative to *oriC* at the *bioA* locus in DY380 cells [4].

Correction-SSOs of different lengths harboring a mismatch at the center were designed to repair the amber codon on the mCAT reporter (Fig. 1). Recombinants are conferred chloramphenicol resistance (CM^r) and the efficiency of recombination can be calculated by dividing the number of CM^r colonies to those that survived electroporation after overnight incubation at 32 °C.

Consistent with our previous findings employing the mKan reporter [14], we observed a general trend for higher efficiency of recombination when SSO lengths increased (Table 1). The *E. coli* methyl-directed mismatch repair (MMR) system is known to repair single nucleotide mismatches with differential efficiencies [16,17], and accordingly, the MMR defective strain, i.e., *mutS*, displayed elevated frequencies of recombinant formation when different mismatch is imposed by the correction-SSO at the mCAT reporter (Table 1). Moreover, in agreement with the role of replication in dictating strand bias, SSOs that correspond in sequence to the nascent lagging strand were more competent in directing formation of CM^r colonies than their respective complementary SSOs. To further generalize these findings, the mKan reporter was integrated in both orientations relative to *oriC* at the *recA* locus in DY330 cells. Similar to the above results obtained from the mCAT chromosomal reporters, SSO mutagenesis using the mKan reporters at the *recA* locus followed the same trends (Table 2). Our observations that λ-Red mediates

SSO	DNA Sequence
89C	5′-CAC AAG TTT TA**C** CCG GGC TTT-3′
89T	5′-CAC AAG TTT TA**T** CCG GGC TTT-3′
mCM reporter	5′-CAC AAG TTT TAG CCG GGC TTT-3′ 3′-GTG TTC AAA ATC GGC CCG AAA-5′
89G	3′-GTG TTC AAA AT**G** GGC CCG AAA-5′
89A	3′-GTG TTC AAA AT**A** GGC CCG AAA-5′

Fig. 1. Sequence near the mCM amber TAG. Correction-SSOs each with a single base mismatch to convert the amber codon to a tyrosine residue is bolded.

Table 1
Rates of CM^r recombinant formation

DY380mCM(+)	WT	Δ*mutS*	DY380mCM(+)	WT	Δ*mutS*
89C lagging	1.4570 ± 0.2518	2.1970 ± 0.3231	89G leading	0.0198 ± 0.0030	0.1163 ± 0.0119
37C lagging	1.4280 ± 0.1830	2.9490 ± 0.6595	37G leading	0.0297 ± 0.0028	0.3182 ± 0.1211
23C lagging	0.1571 ± 0.0387	0.1252 ± 0.04446	23G leading	0.0712 ± 0.0133	0.0603 ± 0.0079
15C lagging	0.0187 ± 0.0093	0.0024 ± 0.0011	15G leading	0.0010 ± 0.0002	0.0002 ± 0.0001
DY380mCM(−)	WT	Δ*mutS*	DY380mCM(−)	WT	Δ*mutS*
89G lagging	0.2654 ± 0.0210	1.5660 ± 0.4730	89C leading	0.1269 ± 0.0425	0.0139 ± 0.0038
37G lagging	0.4591 ± 0.0677	2.1210 ± 0.0972	37C leading	0.1470 ± 0.0213	0.0343 ± 0.0193
23G lagging	0.2721 ± 0.0507	1.4520 ± 0.3703	23C leading	0.0104 ± 0.0018	0.0046 ± 0.0012
15G lagging	0.0127 ± 0.0020	0.0817 ± 0.0143	15C leading	0.0001 ± 0.0001	0.0007 ± 0.0004

λ-Red-induced wildtype (WT) and MMR-defective (Δ*mutS*) DY380 cells harboring a mCAT gene reporter were electroporated with correction-SSOs of different lengths. Figures present means ± SEM of the percentage of CM^r colonies that formed after overnight incubation at 32 °C.

Table 2
Rates of kan^r recombinant formation

DY330mkan(+)	WT	Δ*mutS*	DY330mkan(+)	WT	Δ*mutS*
8A lagging	3.5150 ± 0.5660	3.0530 ± 0.5839	8B leading	0.0284 ± 0.0064	0.3369 ± 0.0992
6A lagging	0.3699 ± 0.0642	0.4181 ± 0.0761	6B leading	0.0461 ± 0.0117	0.2838 ± 0.0718
2A lagging	0.0150 ± 0.0036	0.0187 ± 0.0037	2B leading	0.0013 ± 0.0005	0.0158 ± 0.0043
DY330mkan(−)	WT	Δ*mutS*	DY330mkan(−)	WT	Δ*mutS*
8B lagging	0.1350 ± 0.0498	3.5870 ± 0.8950	8A leading	0.5036 ± 0.1659	0.4589 ± 0.0941
6B lagging	0.1051 ± 0.0302	1.9050 ± 0.4225	6A leading	0.1761 ± 0.0418	0.3108 ± 0.0839
2B lagging	0.0017 ± 0.0009	0.1200 ± 0.0401	2A leading	0.0018 ± 0.0006	0.0067 ± 0.0021

λ-Red-induced wildtype (WT) and MMR-defective (Δ*mutS*) DY330 cells harboring a mKan resistance gene reporter were electroporated with correction-SSOs of different lengths. Figures present means ± SEM of the percentage of CM^r colonies that formed after overnight incubation at 32 °C.

recombinant formation in the two antibiotic resistance gene reporters (mCAT and mKan) at different chromosomal loci (*bioA* vs. *recA*) in different bacterial strains (DY380 vs. DY330) illustrate general applicability of the system in promoting DNA sequence modifications. Together, our findings further substantiate the role of the direction of replication in determining strand bias between a SSO and its chromosomal target during λ-Red mediated recombination.

To evaluate λ-Red-mediated recombination between a SSO and its plasmid target, a pair of plasmids that harbor the mCAT gene was assembled and served as reporters to assess the rate of recombination events. These plasmid substrates were first established as stable episomes in DY380 cells. Overnight cultures were expanded and induced to express λ-Red functions prior to electroporation with different SSOs. Similar to our experiments using the chromosomal reporters, we also evaluated the frequencies of CM^r colony formation in a MMR defective DY380 strain, i.e., Δ*mutS*.

As seen in Fig. 2A, SSOs with the longer lengths of homology directed recombination at higher rates than those with shorter lengths of homology to the target DNA sequence. Moreover, strand bias was observed that favored SSOs with sequence corresponding to the nascent lagging strand.

To exclude a mismatch-specific effect, we designed SSOs that impose two other mismatches when aligned to its target DNA sequence. Taking advantage of the redundant codons of tyrosine, a A/G or a T/C mismatch can be imposed to revert the amber mutation on the plasmid reporters (Fig. 1). Similar to the results obtained using SSOs that impose a G/G or a C/C mismatch, the 89 and 19 bp SSOs followed a strand bias that is dependent on the direction of replication when MMR function is abolished (i.e., Δ*mutS*; Fig. 2B). The length of homology of the SSOs also displayed a positive effect on the rates of recombination events.

Having established that characteristics of gene targeting events are consistent across different reporter systems, we tested the possibility of a role of transcription in this cellular process. Transcriptional processes have been suggested to mediate oligonucleotide-directed gene repair events in mammalian cells [10,18], however, how transcription affects recombinant formation in prokaryotes remains elusive. To this end, we constructed an analogous reporter system, where the mCAT allele is under the control of the arabinose-inducible promoter. The mCAT sequence was subcloned in both orientations relative to ColE1 to give pGKfrtaramCM(+) and pGKfrtaramCM(−). Expression of the reporters can be induced by the addition of arabinose or repressed by the addition of glucose.

To test the stringency of the arabinose promoter, bacterial cells harboring an inducible episomal CAT gene was spread onto LB agar plates supplemented with 0.4% glucose + 12.5 μg/ml chloramphenicol. In contrast to cells spread onto LB agar plates supplemented with 0.4% arabinose + 12.5 μg/ml chloramphenicol, no growth of colonies

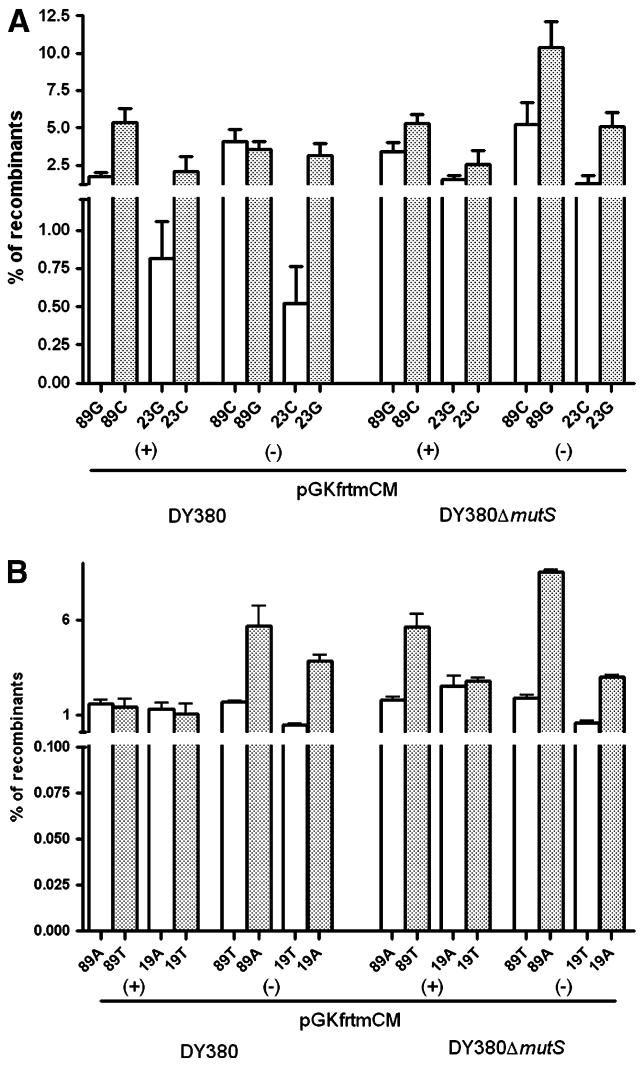

Fig. 2. Strand preference during SSO-mediated repair of mutant episomal reporter. (A) SSOs of 89 and 23 bp. (B) SSOs of 89 and 19 bp were used to repair the amber mutation on the mutant CAT gene residing on the pGKfrtmCM reporters. The *y*-axis represents the percentage of recombinants that formed relative to the total number of electroporation-surviving cells. Both the wildtype DY380 and the MMR-defective derivative (DY380ΔmutS) were tested. The empty and shaded bars represent the yielded percentage of recombinant formation using SSO corresponding in sequence to the nascent leading and lagging strands, respectively.

was observed (data not shown), illustrating that repressed expression of the reporters cannot support growth of bacterial cells, presumably due to low levels of transcription of CAT gene.

Overnight cultures of DY380 cells harboring either the pGKfrtaramCM(+) or the pGKfrtaramCM(−) plasmids were tested essentially as above except that the cells were incubated in either 0.4% arabinose to induce expression of the mCAT allele, or 0.4% glucose to repress transcription of the mutant reporter. The bacterial cells were made electrocompetent after λ-Red induction. Thereafter, SSOs were introduced to restore the mutant antibiotic gene. Transformed cells were incubated for an additional 2 h in LB medium supplemented with either 0.4% arabinose or 0.4% glucose before being spread onto LB agar plates supplemented with 0.4% arabinose + chloramphenicol.

In the presence of arabinose, SSO of 89 and 37 bp directed recombination with a strand bias that favored the lagging SSOs (Table 3). To test whether transcriptional processes affect recombinant formation, the SSOs were similarly introduced into bacterial cells that were incubated in the presence of 0.4% glucose to repress transcription of the reporter.

When expression of the reporter is repressed, complementary SSOs restored the CAT gene with strand preference resembling that of the actively transcribed reporters (Table 3). The fact that we did not observe any difference in strand bias when transcription level varied suggests that strand preference is largely independent of transcriptional processes. Despite the negligible change in strand bias, consistent with our previous findings employing the IPTG inducible plasmid reporters [5,14], we observed significantly higher rates of recombinant formation when transcription is activated. Moreover, enhancement of recombinant formation ranged from 2- to 10-folds, depending on the length of Correction-SSO used.

To monitor the maximal promoting effects of transcriptional processes during λ-Red/SSO-mediated recombination, we repeated our experimentations using the inducible mCAT reporter essentially as above except that transformed bacterial cells were incubated in glucose for different lengths of time prior to plating onto arabinose-supplemented LB agar.

When SSO 89C or SSO 89G was used to restore the functional CM^r gene residing on pGKfrtaramCM(+), we observed a reproducible but insignificant trend of decreasing recombination rates over the time course (Fig. 3A). Strikingly, prolonged repressed transcription resulted in significant decrease in the frequencies of recombination when SSO 15C and 15G were used (Fig. 3B). The reduction was also found to stabilize after 3 h of glucose incubation, suggesting that recombination events were completed during the initial 3 h after SSO introduction.

When SSO 89C or SSO 89G was electroporated into recombinogenic DY380/pGKfrtaramCM(−) cells, a small but significant decrease in recombination frequency was observed when transcription is repressed (Fig. 3C). Similarly, SSO 15C and SSO 15G also resulted in significant reduction in recombination rates when transformed cells were incubated in glucose for a prolonged period (Fig. 3D).

Discussion

We previously reported a role of replication and mismatch repair in determining strand bias and gene repair efficiency during λ-Red/SSO-mediated recombination [5,14]. To consolidate this finding, a mCAT reporter system was similarly constructed to facilitate evaluation of recombinant formation. We also adopted our previously described mutant kanamycin resistance gene to generate a pair of chromosomal reporters at the *recA* locus on the DY330 *E. coli*

Table 3
Active transcription promotes recombinant formation

Lagging	+Arabinose	+Glucose	Leading	+Arabinose	+Glucose
DY380/pGKfrtaramCM(+)					
89C	3.684 ± 0.674	0.889 ± 0.155	89G	0.595 ± 0.101	0.187 ± 0.049
37C	5.677 ± 0.978	0.522 ± 0.089	37G	2.611 ± 0.411	0.243 ± 0.046
15C	0.060 ± 0.021	0.014 ± 0.003	15G	0.135 ± 0.0443	0.037 ± 0.007
Leading	+Arabinose	+Glucose	Lagging	+Arabinose	+Glucose
DY380/pGKfrtaramCM(−)					
89C	0.822 ± 0.186	0.475 ± 0.096	89G	1.395 ± 0.255	0.494 ± 0.071
37C	3.181 ± 0.533	0.448 ± 0.082	37G	4.183 ± 0.591	0.523 ± 0.060
15C	0.022 ± 0.012	0.010 ± 0.003	15G	0.454 ± 0.123	0.124 ± 0.031

λ-Red-induced DY380 cells harboring either pGKfrtaramCM plasmid reporters were electroporated with correction-SSOs. Transformed cells were recovered either in LB medium supplemented with 0.4% arabinose or 0.4% glucose to induce or repress transcription, respectively. Thereafter, the cells were plated onto LB agar plates supplemented with 0.4% arabinose. Figures present means ± SEM of the percentage of CM^r colonies that formed after overnight incubation at 32 °C.

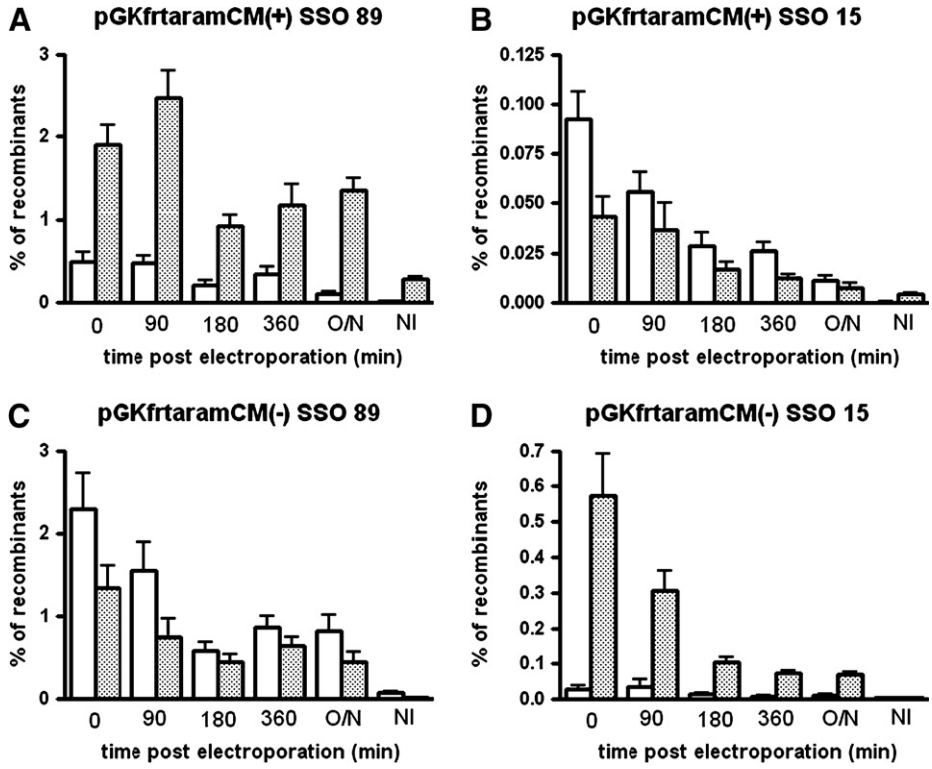

Fig. 3. Time course of repressed transcription during λ-Red/SSO-mediated recombination. SSOs of (A) 89 bp and (B) 15 bp, and (C) 89 bp and (D) 15 bp were used to repair the mutant CAT reporter pGKfrtaramCM(+) and pGKfrtaramCM(−) driven by the arabinose-inducible promoter, respectively. λ-Red-induced DY380 cells were electroporated with Correction-SSOs prior to incubating in LB medium supplemented with 0.4% glucose for different time intervals (*x*-axis; 0, 90, 180, 360 min and O/N; overnight) to repress transcription of the reporter gene. Transformed cells were subsequently plated onto LB agar plates containing 0.4% arabinose to induce expression of the reporter. The *y*-axis represents the number of CM^r colonies that formed after overnight incubation at 32 °C relative to the number of cells that survived electroporation. The percentage of recombinant formation of non-induced cells (NI) is also included. The empty and shaded bars represent the yielded percentage of recombinant formation using SSO corresponding in sequence to the nascent leading and lagging strands, respectively.

chromosome [5], thus allowing strain- and chromosomal locus-specific effect to be excluded from our results.

Consistent with the current understanding of the phage-promoted recombination process, we found that the length of homology on Correction-SSOs exhibited a positive effect on the overall recombination efficiencies when targeted to both chromosomal and episomal loci. Moreover, strand preference for chromosomal target correction favored SSOs that correspond in sequence to the nascent lagging strand. This is true for both mCAT and mKan reporter

systems at either the *bioA* or the *recA* locus. Our findings here not only are consistent with the notion that the direction of replication dictates strand bias, but further strengthen the implicated role of replication in λ-Red/SSO-mediated recombination [4].

Collision of replication and transcription machineries has been a subject of research in both prokaryotes and eukaryotes [19–21]. These machineries have adopted different means to avoid fork collapses despite the conflicts between the two cellular processes [22–24]. Taking into account the short cycle of *E. coli* and that replication plays an intimate role in SSO mutagenesis in *E. coli*, the impact of a converging transcription fork with that of replication is envisioned to affect the ultimate efficiency of targeted repair events. To address this possibility, we moved the mCAT gene under the control of an arabinose inducible promoter. Induction and repression of transcription of the reporter sequence can be easily manipulated by the addition of arabinose and glucose, respectively.

Active transcription of the episomal reporters was found to promote the rate of recombinant formation with both mCAT and mKan reporters. It is possible that collision between the replication and transcription complexes elevates the accessibility of the SSO-β nucleoprotein filament to its target sequence by slowing down replication fork progression [20,24]. Similar to this idea, thymine-induced slow down of replication fork progression was recently shown to enhance gene repair events in a mammalian model [25]. Likewise, transcription activity has also been demonstrated to elevate frequency of gene repair events in yeast [18]. Whether the observed increase in gene repair events is due to a relaxed DNA structure during active transcription or a slow down of replication fork, or both, warrants further investigation. Nevertheless, active transcription of the plasmid reporters described in this study resulted in as much as a 10-fold increase in the rates of SSO-dependent recombinant formation, further implicating a role of transcriptional processes during targeted gene repair in *E. coli*.

Collectively, our results not only provide additional evidence for the implicated role of replication during λ-Red/SSO-mediated recombination in *E. coli*, but also reveal a role of transcription in the targeted gene repair process. The nature of the interplay of replication and transcription during SSO mutagenesis awaits further studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.11.146.

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