

# Enhanced levels of $\lambda$ Red-mediated recombinants in mismatch repair mutants

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**Homologous recombination can be used to generate recombinants on episomes or directly on the *Escherichia coli* chromosome with PCR products or synthetic single-stranded DNA (ssDNA) oligonucleotides (oligos). Such recombination is possible because bacteriophage  $\lambda$ -encoded functions, called Red, efficiently recombine linear DNA with homologies as short as 20–70 bases. This technology, termed recombineering, provides ways to modify genes and segments of the chromosome as well as to study homologous recombination mechanisms. The Red Beta function, which binds and anneals ssDNA to complementary ssDNA, is able to recombine 70-base oligos with the chromosome. In *E. coli*, methyl-directed mismatch repair (MMR) can affect these ssDNA recombination events by eliminating the recombinant allele and restoring the original sequence. In so doing, MMR can reduce the apparent recombination frequency by >100-fold. In the absence of MMR, Red-mediated oligo recombination can incorporate a single base change into the chromosome in an unprecedented 25% of cells surviving electroporation. Our results show that Beta is the only bacteriophage function required for this level of recombination and suggest that Beta directs the ssDNA to the replication fork as it passes the target sequence.**

**H**omologous recombination mediated by  $\lambda$  Red has been used as a genetic tool to modify the bacterial chromosome with linear double-stranded DNA (dsDNA) (1–6). In addition to linear dsDNA, single-stranded DNA (ssDNA) oligonucleotides (oligos) have been used to modify the chromosomes of both yeast and *Escherichia coli* (7–9). In yeast, the functions are not yet defined that allow oligo recombination; however, in *E. coli*, the bacteriophage  $\lambda$  Red recombination system is involved (9).

The  $\lambda$  Red system includes the Gam, Exo, and Beta proteins. Whereas Red-mediated recombination between linear duplex DNA and the bacterial chromosome requires all three functions (1, 3, 10), recombination with ssDNA oligos requires only the Beta protein (9). Beta protein binds stably to ssDNA (11) >35 nt in length (12), protects it from single-strand nuclease attack (13, 14), and promotes annealing to complementary ssDNA (13–15).

Beta binds ssDNA from 3' to 5' (13, 16) but does not bind directly to dsDNA (13, 14). However, after Beta generates dsDNA by annealing two complementary ssDNAs, it remains tightly bound to the annealed dsDNA (13, 14, 16). As it anneals strands to form the dsDNA, it generates DNA filaments similar to those formed by RecA–DNA complexes (17). This annealed dsDNA–Beta complex is resistant to DNase I and is much more stable than the ssDNA–Beta complex (16).

Although Beta can catalyze single-strand annealing, it cannot promote strand invasion of a duplex DNA with a homologous ssDNA during recombination (16, 18). Therefore, Beta-mediated recombination with ssDNA is likely to occur by annealing with transiently single-stranded regions of the chromosome. Initial results suggest that Beta-dependent ssDNA recombination may occur at the DNA replication fork. When either of the two complementary ssDNA oligos for a specific position in the chromosome is used for recombination, the

oligo that corresponds in sequence to Okazaki fragments always generates the highest efficiency (9, 10). In this article, we will refer to such oligos as the lagging-strand oligo and their complement as the leading-strand oligo.

Many host proteins are involved in the progression of the replication fork. Here, we focus on replication proofreading by the methyl-directed mismatch repair (MMR) system. The host MMR system corrects base incorporation errors that occur during replication by first recognizing and then excising the incorrect base (19–23). If the ssDNA oligo used for recombineering introduces a change near the DNA replication fork, this change may trigger mismatch repair, which in turn can affect the outcome of recombination (24–26). Our results demonstrate that defects in the MMR system can enhance the yield of recombinants generated with Red-mediated recombineering by >100-fold; under some conditions used, nearly one-quarter of all cells contain the desired base change.

## Materials and Methods

**Genotype of Strains.** HME5 is W3110  $\Delta$ (*argF-lac*)U169 *gal*<sup>+</sup>{ $\lambda$ cI857 $\Delta$ cro-bioA}. HME6 is HME5 *galK*<sub>TYR145UAG</sub>, HME31 is HME5 *galK*<>*cat-sacB*, and HME41 is HME6 with the entire *galETKM* operon inverted with respect to the adjacent genes. HME43 is a derivative of HME6 in which *bet* is present but the *exo* and *gam* genes have been deleted. These strains have previously been described (9). MMR mutant derivatives of these strains are defined below and in Tables 1–4, where they are used. HME6, HME41, HME43, and MMR mutant derivatives were made *recA*<sup>−</sup> by P1 transduction to move in  $\Delta$ srI-*recA*::Tn10.

**Deletion of Host Factor Genes.** Host MMR genes *mutH*, *mutL*, *mutS*, *uvrD*, and *dam* were deleted by inserting PCR generated antibiotic resistance cassettes in place of the coding region of each MMR gene in strain HME6. Either a kanamycin (*kan*) or ampicillin (*amp*) resistance cassette was amplified by PCR by using oligos that contained at their 5' ends 45–55 bases of homology flanking the target MMR gene (see below). The PCR cassettes with flanking homology were introduced by Red-mediated recombination; recombinants were selected for antibiotic resistance and verified by analytical PCR (3). Once the substitution was confirmed, it was moved by P1 transduction into the appropriate strains.

Schematic descriptions of the PCR amplified *kan* and *amp* cassettes are indicated below for each MMR gene. The sequences in capital letters, which flank the drug cassettes, define part of the 45–55 bases of homology for chromosomal regions within or adjacent to the designated MMR gene. The antibiotic primer sequences at the 3' end of these oligos (not shown) are as defined for *kan* and *amp* by Yu *et al.* (3).

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

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OLIGO 100 CAG CTT TAt CAT CTG  
 OLIGO 144 CAG CTT TAc CAT CTG  
 5' CAG CTT TAG CAT CTG  
 3' GTC GAA ATG GTA GAC  
 OLIGO 101 GTC GAA ATa GTA GAC  
 OLIGO 145 GTC GAA ATg GTA GAC

**Fig. 1.** Sequence of *galK* amber allele and oligos. The sequence of part of both strands of the *galK* amber gene is shown centered on the TAG amber codon. The G:C pair to be changed is boxed. Part of the 70-base oligo sequence for the four oligos used to correct the amber codon back to tyrosine codons TAT or TAC is shown above and below the gene sequence, with the base change shown in lower case.

*mutH*<>*amp* = (5'*mutH*... AGGTATCATGAC-*amp*-AGTGCACTACTG... 3'*mutH*)  
*mutL*<>*amp* = (5'*mutL*... CAACTGGCGAAC-*amp*-TACATCCGGCGA... 3'*mutL*)  
*mutS*<>*amp* = (5'*mutS*... GGACATAACCCC-*amp*-TAATAACAATTC... 3'*mutS*)  
*uvrD*<>*kan* = (5'*uvrD*... GGACGTTTCTTA-*kan*.-TACGTTGCCCCG... 3'*uvrD*)  
*dam*<>*kan* = (5'*dam*... AGGGGGCAAGTA-*kan*-TTCTCAAGGAGA... 3'*dam*)

**Materials.** Oligos were supplied by Invitrogen as salt-free but otherwise unpurified. All ssDNA oligos used to correct the *galK* mutations were 70 bases in length. The sequence of Oligo 100, which corrects the TAG stop to a TAT tyrosine codon, is: 5'-AAGTCGCGGTTCGGAACCGTATTGCAGCAGCTTTATCATCTGCCGCTGGACGGCGCACAAATCGCGCTTAA-3'. Oligo 101 is the complement to 100. Oligos 144 and 145 are identical to 100 and 101, respectively, except that they correct the TAG stop to a TAC tyrosine codon (Fig. 1). Invitrogen supplied *Taq* polymerase HiF and Concert Rapid PCR purification kits. The following 71-base oligo was used to mutate the *malK* gene to an amber codon replacing the TAC tyrosine codon at position 84:

5'-ATGTTTTCTGCTACTGACAGGTGGGGATAGAGCGCCTAAGACTGAAACACCATACCAACGCCGCTTCTGC.

**Recombination Assays for Galactose (Gal) Phenotypes.** Strains were induced at 42° for 15 min to express λ Red functions and immediately made electrocompetent as described (27, 28). A saturating level of each ssDNA oligo (5 pmol) was used per electroporation. Gal<sup>+</sup> recombinant colonies were selected on M63 minimal galactose plates with biotin, and viable cells were counted on LB agar as described (9). Gal<sup>-</sup> recombinant colonies from a *gal*<sup>+</sup> derivative of HME6*mutS* were selected on 2-deoxygalactose as described (3, 9). The percentage of cells that survive electroporation in the HME6 background used in these studies is ≈5–10% and is independent of oligo addition or prophage induction.

The *gal* operon transcription was induced by adding 0.2% fucose to LB medium.

**Nonselective Analysis of Recombinants.** After electroporation, cells were diluted and spread on LB agar or diluted into 10 ml of LB and grown for 1, 2, or 3 h at 32°C before being further diluted and spread on LB agar to examine viable cells. Individual colonies from LB agar were tested for the presence of Gal<sup>+</sup>

**Table 1.** Oligonucleotide recombination efficiency

Oligo <sup>†</sup>	Gal <sup>+</sup> recombinant per 10 <sup>8</sup> viable cells*	
	<i>galK</i> <sub>TYR145UAG</sub> <sup>‡</sup>	<i>galK</i> <> <i>cat-sacB</i> <sup>‡</sup>
100 (T-C)	3.5 × 10 <sup>5</sup> (26)	8.6 × 10 <sup>4</sup> (13)
101 (A-G)	4.8 × 10 <sup>4</sup> (20)	2.8 × 10 <sup>3</sup> (15)
144 (C-C)	3.1 × 10 <sup>7</sup> (3)	1.2 × 10 <sup>5</sup> (2)
145 (G-G)	1.8 × 10 <sup>4</sup> (3)	2.3 × 10 <sup>3</sup> (2)

\*Strain HME6 has point mutation (*galK*<sub>TYR145UAG</sub>), and HME31 has *cat-sacB* insertion.

<sup>†</sup>The relevant part of the oligo sequences is shown in Fig. 1. The mismatch pair (in parentheses) is created by annealing the oligo at *galK*<sub>TYR145UAG</sub>.

<sup>‡</sup>Results are presented as an average from several independent experiments (number in parentheses). The standard deviation from the mean was ≈50%.

recombinants by patching to MacConkey galactose indicator agar and scoring for a red color. Cells from the electroporation mixture were also diluted and spread directly on MacConkey galactose indicator agar to identify and examine individual recombinant colonies directly. Cells derived from the recombination process form recombinant red (Gal<sup>+</sup>) and nonrecombinant “white” or colorless (Gal<sup>-</sup>) colonies. Sectoring red and white colonies indicate the segregation of recombinant chromosomes in the original recombinant cell. Similar tests for *malK*<sub>TYR84UAG</sub> recombinants were carried out on MacConkey maltose agar except in this case the recombinants were Mal<sup>-</sup> white colonies.

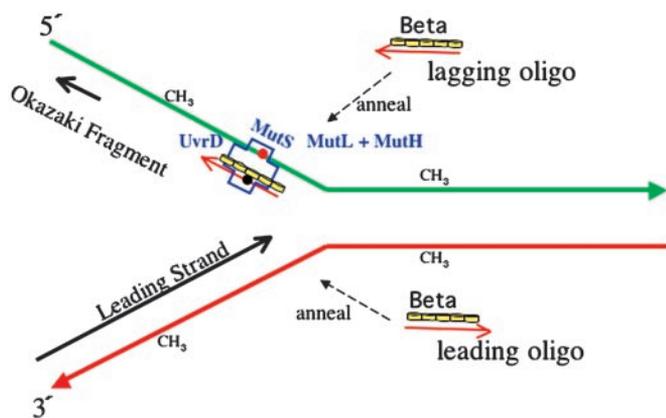
## Results

**An Oligo Sequence Affects Recombination Efficiency.** The *galK*<sub>TYR145UAG</sub> mutation is a single-base change at codon 145 in the *galK* gene creating an amber codon (3). Another mutation *galK*<>*cat-sacB* is an insertion at the same position as the amber point mutation (9). Recombination with oligo 100, whose partial sequence is in Fig. 1, has been shown to correct both mutations with nearly the same efficiency (9).

Oligo 100 and its complement 101 are designed to correct the *galK*<sub>TYR145UAG</sub> to the original TAT tyrosine codon. Two other oligos, oligo 144 and its complement 145, are designed to correct the *galK* mutation to the other tyrosine codon, TAC (Fig. 1). As described (9), oligo 100 or 101 efficiently recombines to generate Gal<sup>+</sup> recombinants with *galK*<sub>TYR145UAG</sub> (Table 1). Oligo 145 generated a similar level of Gal<sup>+</sup> recombinants. However, the frequency of Gal<sup>+</sup> recombinants with oligo 144 was ≈100-fold greater than for oligo 100 despite the fact that oligos 100 and 144 differ by only the one base at the tyrosine codon. The other sequence related oligo pair of 101 and 145 had similar frequencies of Gal<sup>+</sup> recombinants (Table 1).

Each of the four oligos was also tested for recombination efficiency with the *galK*<>*cat-sacB* insertion. Unlike the result with the point mutation, oligos 100 and 144 recombined to generate Gal<sup>+</sup> from the *cat-sacB* allele with nearly the same efficiency (Table 1). Likewise, oligos 101 and 145 produced similar numbers of recombinants but less than for oligos 100 and 144. Thus, oligo 144 yields a dramatic increase in recombinants formed with the *galK*<sub>TYR145UAG</sub> point mutation, but the same enhancement is not provided during recombination with the 3.3-kbp *cat-sacB* heterology in *galK*. This result suggests that the pairing difference of oligos 100 and 144 (see Fig. 1) with the complementary strand of the *galK*<sub>TYR145UAG</sub> mutation might cause the difference in recombination efficiencies observed.

Our previous results have suggested that Red-mediated oligo recombination is likely to occur at the DNA replication fork (Fig. 2) based on the fact that the oligo corresponding in



**Fig. 2.** Repair of mismatched base pair at replication fork. A replication fork is shown in which a lagging-strand oligo (short red arrow) has been annealed to a gapped single-strand region by Beta. The leading- and lagging-strand regions are shown. An Okazaki fragment is indicated on the lagging strand. MutS is shown bound to the mismatch created at the position of the annealed oligo. MutL, MutH, and UvrD helicase are shown ready to assemble. Methyl (CH<sub>3</sub>) groups are shown on parental strands. Arrows are shown in the 5' to 3' direction. The leading and lagging oligos with Beta bound are shown only for illustration and would not normally be in the same cell for experiments like those described here.

sequence to the Okazaki fragments is always more efficient at recombination than the complementary oligo (9, 29). If paired at the replication fork, the oligo 100 TAT sequence would generate a T·C mismatch with the parental strand, whereas the oligo 144 TAC sequence generates a C·C mismatch at the same position (Fig. 1). The MutS protein of the MMR system binds and in conjunction with other MMR functions corrects a T·C mismatch as well as most other single-base pair mismatches or one to three nucleotide insertions. However, the MMR system fails to repair a C·C mismatch (30). The MMR system also does not recognize mismatches created between large heterologies like those expected for *cat-sacB* repair by the oligos (31). Thus, the efficiency of repair of *cat-sacB* by these oligos may directly reflect the recombination rate, whereas, with the *galK* amber mutant, the recombinant yield may be reduced 100-fold by MMR.

**Mismatch Repair Functions Are Important in Correcting *galK*<sub>TYR145UAG</sub> but Not *cat-sacB*.** Several mutations causing a defect in the MMR system were generated in strains HME6 (*galK*<sub>TYR145UAG</sub>) and HME31 (*galK* $\leftrightarrow$ *cat-sacB*) and then tested for their effect on oligo-mediated recombination. Elimination of the *mutH*, *mutL*, *mutS*, or *uvrD* genes in HME6 increased oligo-mediated recombination 25- to 60-fold relative to wild type (Table 2). We also tested a Dam methylase mutant, *dam* $\leftrightarrow$ *kan*. In the HME6 *dam* $\leftrightarrow$ *kan* strain, a 5- to 10-fold increase in recombination efficiency was observed relative to HME6. The MMR-deficient strains did not show a difference in recombination frequency when oligos 100 and 101 were used to remove the large heterology *cat-sacB* (Table 2). These results support the inference that MMR reduces the number of recombinants generated by oligos, and that this reduction is specific for single base changes. Results in Table 3 show that MMR reduces recombination for all oligos except 144, which introduces the C·C mismatch.

Beta is the only phage protein required for oligo-mediated recombination (9, 10). We found that Beta was sufficient to provide the high levels of recombination found in the *mutS*-defective strain (Table 3). Additionally, the high levels of recombination are independent of RecA (data not shown).

**Table 2. Effect of MMR genes on recombination with *galK* point and insertion mutations**

MMR	Gal <sup>+</sup> recombinants per 10 <sup>8</sup> viable cells			
	<i>galK<sub>am</sub></i>		<i>galK</i> $\leftrightarrow$ <i>cat-sacB</i>	
	100	101	100	101
Allele*				
<i>mut</i> <sup>+</sup>	4.7 × 10 <sup>5</sup>	2.4 × 10 <sup>4</sup>	1.2 × 10 <sup>5</sup>	4.0 × 10 <sup>3</sup>
$\Delta$ <i>mutH</i>	2.1 × 10 <sup>7</sup>	5.8 × 10 <sup>5</sup>	5.2 × 10 <sup>4</sup>	1.7 × 10 <sup>3</sup>
$\Delta$ <i>mutL</i>	2.2 × 10 <sup>7</sup>	7.5 × 10 <sup>5</sup>	1.0 × 10 <sup>5</sup>	9.0 × 10 <sup>2</sup>
$\Delta$ <i>mutS</i>	3.6 × 10 <sup>7</sup>	1.5 × 10 <sup>6</sup>	1.4 × 10 <sup>5</sup>	3.3 × 10 <sup>3</sup>
$\Delta$ <i>uvrD</i>	2.7 × 10 <sup>7</sup>	1.6 × 10 <sup>6</sup>	†	†
$\Delta$ <i>dam</i>	2.7 × 10 <sup>6</sup>	2.6 × 10 <sup>5</sup>	2.3 × 10 <sup>5</sup>	2.5 × 10 <sup>3</sup>

\*Spontaneous reversion to a Gal<sup>+</sup> phenotype occurs at a frequency of  $\approx$ 1 per 10<sup>8</sup> cells of strain HME6 *mut*<sup>+</sup> and 24 per 10<sup>8</sup> cells of strain HME6 *mutS*.

†Strain has not been made.

**Increasing the Yield of Oligo-Generated Recombinants by 2-Aminopurine (2-AP).** We have shown that MMR mutants enhance the yield of oligo-generated recombinants. Because MMR-defective strains are mutagenic (20), we attempted to stimulate recombination by transiently inhibiting MMR activity. Haber and Walker (32) have shown that induction of a dominant negative allele of MutS can cause a temporary MMR-defective state *in vivo*. Two other treatments have been shown to titrate MutS or MutL and reduce MMR activity. MutS protein can be titrated by the mismatched bases that are present in covalently joined DNA-RNA molecules produced by retron-like elements in *E. coli* (33). MutL protein appears to be limited when cells are treated with the adenine analog 2-AP (34), because the analog incorporates and mispairs with cytosine during DNA replication. These conditions cause a functionally defective MMR system during the course of treatment. Using the conditions set forth previously (34), we found that incubation times with 2-AP of <2 h showed little if any effect on recombination. However, 3 h of incubation in LB with 75  $\mu$ g/ml 2-AP present increased the number of oligo100-directed Gal<sup>+</sup> recombinants in the MMR proficient HME6 strain by  $\approx$ 10-fold to 2–5 × 10<sup>6</sup> per 10<sup>8</sup> survivors. Although this is  $\approx$ 10-fold less than that observed in the *mutS* mutant, it demonstrates that a temporary inhibition of mismatch repair can be induced to enhance recombination levels for specific recombination experiments as indicated by Matic *et al.* (34). We also coelectroporated a 70-bp dsDNA, lacking homology to *E. coli* and containing a G·G mismatch with oligo 100 but failed to see any effect of the mismatched oligo pair on recombination frequencies even when it was in 100-fold excess (data not shown).

**The DNA Strand Recombination Bias Caused by Replication Is Independent of Oligo Sequence and Mismatch Repair Process.** Ellis *et al.* (9) demonstrated that a strand bias observed in oligo-mediated recombination depends on the direction of DNA replication

**Table 3. Beta function supports full ssDNA recombination**

Oligo	Gal <sup>+</sup> recombinants per 10 <sup>8</sup> cells*	
	HME43 <i>mut</i> <sup>+</sup>	HME43 <i>mutS</i> <sup>†</sup>
100 (T·C)	2.9 × 10 <sup>5</sup>	1.6 × 10 <sup>7</sup> (55)
101 (A·G)	4.4 × 10 <sup>4</sup>	1.0 × 10 <sup>6</sup> (23)
144 (C·C)	1.2 × 10 <sup>7</sup>	1.3 × 10 <sup>7</sup> (1)
145 (G·G)	2.6 × 10 <sup>3</sup>	1.0 × 10 <sup>6</sup> (385)

\*Strain HME43 is *galK<sub>am</sub>* and deleted for *red* genes *exo* and *gam* (9).

†Fold increase in recombination by deleting *mutS* is indicated in parentheses.

**Table 4. Recombination of leading- or lagging-strand oligos**

Oligo	Gal <sup>+</sup> recombinants per 10 <sup>8</sup> cells*			
	MMR proficient		MMR deficient <sup>†</sup>	
	Lagging strand	Leading strand	Lagging strand	Leading strand
100 (T-C)	<b>4.7 × 10<sup>5</sup></b>	<b>1.1 × 10<sup>4</sup></b>	3.6 × 10 <sup>7</sup> (77)	<b>1.4 × 10<sup>6</sup></b> (127)
101 (A-G)	<b>1.6 × 10<sup>6</sup></b>	<b>4.8 × 10<sup>4</sup></b>	<b>5.5 × 10<sup>7</sup></b> (34)	2.1 × 10 <sup>6</sup> (47)
144 (C-C)	5.2 × 10 <sup>7</sup>	<b>1.3 × 10<sup>6</sup></b>	5.1 × 10 <sup>7</sup> (1)	<b>8.9 × 10<sup>5</sup></b> (1)
145 (G-G)	<b>8.1 × 10<sup>4</sup></b>	1.0 × 10 <sup>4</sup>	<b>3.0 × 10<sup>7</sup></b> (370)	2.1 × 10 <sup>6</sup> (210)

\*Bolted values were obtained in strain HME41 or HME41 *mutS*, in which the *gal* operon is inverted relative to the otherwise identical strains HME6 or HME6 *mutS*, respectively. MMR-proficient strains are HME6 and HME41; MMR-deficient strains are *mutS*. By using *mutS*, the effects of different MMR efficiencies for each mismatch on recombination levels are eliminated. By using normal and inverted *gal* operons, each oligo is tested as a leading or lagging strand relative to the direction of replication through *gal*.

<sup>†</sup>The fold increase in recombination observed by deleting *mutS* is shown in parentheses and indicates the relative efficiency of mismatch correction by the MMR system. C-C mismatches are not corrected by MMR and have a value of 1.

through the target. To further address this model, we used strains HME6 and HME41, both of which contain the *galK*<sub>TYR145UAG</sub>. These strains differ only in the orientation of the *gal* operon, so we can examine the recombination efficiency of each oligo when it acts as either a lagging- or leading-strand oligo. As can be seen in Table 4, each oligo gives a higher recombination frequency when acting as the lagging-strand oligo. However, the variability in lagging-strand recombination is broad, ranging from 8 × 10<sup>4</sup> to 5 × 10<sup>7</sup>, depending on which oligo is used. Variability in recombination efficiency is also observed with the leading strand, where values range from 1 × 10<sup>4</sup> to 1 × 10<sup>6</sup>.

We determined whether these variations would persist in a strain deficient for MMR by testing all four oligos in both HME6*mutS* and HME41*mutS*. In the *mutS* background, the recombination level for lagging-strand oligos, regardless of the mismatch created, was ≈4 × 10<sup>7</sup>. The recombination level with leading-strand oligos was also more uniform, averaging 1.6 × 10<sup>6</sup>. Thus, in the absence of MMR, the oligo-specific effects were eliminated, but the lagging-strand bias remained.

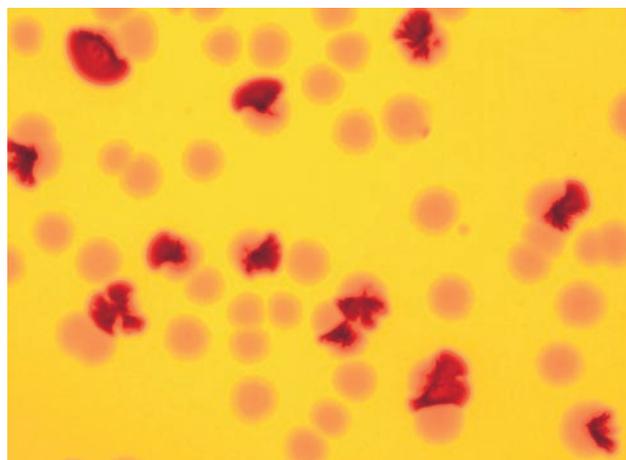
**Nonselective Screening and Segregation of Gal<sup>+</sup> Recombinants.** Red-mediated recombination between an oligo and the *galK* amber allele is astonishingly efficient, as revealed by the frequency of Gal<sup>+</sup> recombinants among cells surviving electroporation when mismatch repair is blocked (Table 2). To determine more precisely the frequency of recombinants and to examine progeny of cells that had undergone recombination, we electroporated HME6 *mutS* cells with oligo100. Cells were immediately spread on MacConkey galactose agar and individual colonies examined. Among colonies that form on MacConkey galactose, Gal<sup>+</sup> (red) and Gal<sup>-</sup> (white) colonies could be easily distinguished. Approximately 80% of the colonies were white nonrecombinants (Gal<sup>-</sup>); the other 20% were white with sectors of red (see Fig. 3). No pure solid red colonies were observed. Because these cells are grown in rich medium before recombination, multiple DNA replication forks result in eight *galK* copies being present in each cell under our conditions (35). Although saturating levels of oligo were used, when recombination occurs in a cell, it is unlikely to occur at every copy of *galK*, and as cells in a colony continue to replicate their DNA and divide, segregation of the recombinant and parental allele occurs, generating mixed or sectorized colonies for the Gal phenotype. If one strand of DNA is recombined, it would be expected to take three generations (3 h) to segregate all of the chromosomes into daughter cells. In fact, we observed uniform red colonies only if the electroporation mixture was incubated in LB for at least 3 h before plating.

Additionally, just after electroporation, the recombination mixture was diluted and spread on LB agar at 32°C for individual colonies. Individual colonies from LB were then patched to MacConkey galactose indicator to determine the fraction of colonies that contained Gal<sup>+</sup> recombinants. This test demonstrated that, in three experiments, 22%, 24%, and 30% of the electroporated cells had undergone recombination, in close agreement with frequencies obtained on MacConkey galactose indicator agar or by selecting for recombinants on minimal galactose agar (see Tables 2–4).

To demonstrate that this observation is not unique to the *galK* gene, we targeted the *malK* gene with an oligo, which generates a C-C mismatch. HME6 mutants defective in the ability to use maltose as a carbon source were found nonselectively at similar high efficiencies of 25–40%, as described above for the Gal<sup>+</sup> recombinants (data not shown). Likewise, starting with a Gal<sup>+</sup> derivative of HME6*mutS*, Gal<sup>-</sup> recombinants were generated with an oligo containing the amber allele (see *Materials*) at this same efficiency.

## Discussion

We demonstrated previously that Red-mediated recombination with synthetic single-strand oligos is very efficient and



**Fig. 3.** MacConkey galactose indicator agar showing recombinant colonies. Cells were spread on MacConkey galactose agar and incubated at 32°C immediately after electroporation of the oligo. White or colorless colonies are from unrecombined parental cells. Colonies with a mixture of red and white patterns are from recombinant cells.

independent of RecA in *E. coli*. Only  $\lambda$  Beta appears to be required for this ssDNA recombination (Table 3) (9, 28, 36). Oligos that correspond to either of the two complementary DNA strands generate recombinants, but invariably one oligo recombines more efficiently than the other. By testing six markers in different regions of the chromosome, a pattern emerged (9). At each position, the most efficient of the two complementing oligos was the one corresponding to the lagging-strand DNA (i.e., the same sequence as the Okazaki fragments). We proposed that oligo-directed recombination occurred at the replication fork, and that the "lagging-strand oligo" is more easily annealed by Beta because of larger gaps present in the lagging strand (9, 29). Three points from the results presented here bear on our previous proposal that Beta-mediated recombination with ssDNA oligos occurs at the replication fork. First, we have demonstrated that four different oligos recombine more efficiently when each is targeted to the lagging strand than when targeted to the leading strand (Table 4). Second, we show that MMR can depress the appearance of recombinants by >100-fold (Table 2). The MMR functions MutS, MutL, MutH, UvrD helicase, and Dam methylase are required for mismatch repair at the replication fork (37). Third, when recombination occurs without bias due to MMR efficiencies, we see incredibly high recombination frequencies of 25%. It is difficult to come up with other mechanisms that generate a single-strand gap at a specific site in 25% of the cells during the time period of the experiment. We do not yet understand why 75% of the cells are resistant to recombination despite saturating levels of oligo. Among several possibilities, some cells may not be electrocompetent, may be in a state resistant to recombination, or may have the target sequestered from the oligo.

Although the lagging-strand oligos generate more recombinants than the leading-strand oligo, the leading-strand oligos are still very recombination proficient. Comparing four oligos (Table 4), lagging-strand recombinants are on average 30-fold more frequent than the leading strand. This may be explained by a proportionally different amount of ssDNA generated during replication on each side of the fork. By this logic, the gaps on the lagging-strand side would be 30 times greater than the gap on the leading-strand side. Of course, other factors could be responsible for this difference, because the type of replication and the factors present at the leading and lagging strands are different (38, 39).

One possible alternative we have explored elsewhere is that transcription generates single-strand regions and affects recombination bias (40). We found that gene transcription does not affect the strand bias observed for oligo-mediated recombination. Our results here strengthen that observation by showing that replication direction is critical to the strand bias.

There are eight possible mismatch pairs, and MutS protein has been shown to bind to each pair *in vitro* (41, 42). The four oligos used here generate four of those eight mismatches. Binding by MutS protein and repair by the MMR system have a similar hierarchical pattern for the eight mismatch pairs (42, 43). The pattern is G·T, A·C, A·A, G·G>T·T, T·C, A·G>C·C, where the C·C mismatch is very weakly bound and poorly corrected. Our studies support a similar pattern of repair efficiency with G·G>T·C>A·G>C·C. A 370-fold difference in repair exists between the well repaired G·G and the poorly repaired C·C mismatches (see lagging strand in Table 4). The same hierarchy and efficiency of correction were found for both the lagging and leading strands.

Because C·C mismatches are not recognized by MMR in other bacterial species (20, 44), this particular feature of oligo recombination might have the potential to create high recombination frequencies in other bacteria. For this reason and others, the ability to transfer the homologous recombination

system to other bacterial species and even to eukaryotes would be very useful. Because ssDNA at the replication fork is bound by Ssb protein (45, 46), and Beta protein is important for the interaction between the oligo and the chromosome target, Beta may interact directly and specifically with Ssb (29). The  $\lambda$  Red system has been shown to work in *Salmonella typhimurium* (47–49), a species very closely related to *E. coli*, and it may also work in other related Gram-negative bacteria. However, for more distantly related bacteria, it may be necessary to provide Beta-like functions from phage endogenous to those bacteria. Exo- and Beta-like proteins have already been identified in other bacterial phages and even eukaryotic viruses such as HSV-1 (50, 51).

Mismatch repair functions are known to prevent DNA exchange between related species by blocking recombination between homologous but divergent sequences (homeologous recombination) (24). A difference between the role of mismatch repair in homeologous recombination and in replication is that in the former, MutS and MutL appear to be required, whereas MutH and UvrD helicase have less importance (24, 25, 52). It is believed that MutS and MutL bind mismatches generated during homeologous exchanges, and the binding itself aborts further recombination without causing repair (53, 54).

Our results suggest the inhibition of oligo-mediated recombination by MMR functions is more analogous to the correction of DNA replication errors than to the role of MMR functions during homeologous recombination. MMR functions tested, including UvrD and MutH, appear to remove mismatches generated during oligo recombination and replication (Table 2). Also, unlike homeologous recombination, Feinstein and Low (55) found that during *E. coli* conjugation between sequences with very few mismatches, the MMR system inhibited recombination and that, like oligo-mediated recombination, MutH and helicase are required. Perhaps, as we suggest for oligo recombination, the incoming strand transferred during normal conjugation is annealed at the replication fork. An alternative is that the recombination intermediates formed generate a new replication fork (39, 56, 57) and recruit the MMR complex. A recent discovery that ssDNA modification of murine embryonic stem cells is inhibited by MMR is consistent with our results and may indicate that in stem cells, the modification is also at the replication fork (58).

The *in vivo* recombination technologies we describe, due to their efficiency, accuracy, and simplicity, may replace classical *in vitro* genetic engineering techniques. The  $\lambda$  Red-mediated homologous recombination system, which we use as a genetic engineering tool, is particularly useful for modifying the genome of *E. coli*, as well as cloned genome segments from other organisms (29, 59, 60). This study creates new opportunities for genome modification and *in vivo* analyses of DNA mechanics. Using synthetic oligos, recombinants with the chromosome and episomes can occur at such high efficiencies that selection is not required. Oligos that create C·C mispairs are recombined into the DNA of cells at efficiencies approaching 25% among the survivors of electroporation. In other words, it might be possible to generate a C replacement of G anywhere in the chromosome at these high efficiencies if the change is not toxic to the cell. Recombination levels approaching 25% were also found for any oligo-generated mismatch in strains defective for the MMR functions tested. This advance in technology allows efficient genetic modifications and may permit nucleotide analogs and adducts to be incorporated directly into the chromosome for *in vivo* biochemical studies.

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