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Methyl-Directed Repair of Frameshift Heteroduplexes in Cell Extracts from *Escherichia coli*

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The methyl-directed DNA repair efficiency of a series of M13mp9 frameshift heteroduplexes containing 1, 2, or 3 unpaired bases was determined by using an in vitro DNA mismatch repair assay. Repair of hemimethylated frameshift heteroduplexes in vitro was directed to the unmethylated strand; was dependent on MutH, MutL, and MutS; and was equally efficient on base insertions and deletions. However, fully methylated frameshift heteroduplexes were resistant to repair, while totally unmethylated substrates were repaired with no strand bias. Hemimethylated 1-, 2-, or 3-base insertion and deletion heteroduplexes were repaired by the methyl-directed mismatch repair pathway as efficiently as the G · T mismatch. These results are consistent with earlier in vivo studies and demonstrate the involvement of methyl-directed DNA repair in the efficient prevention of frameshift mutations.

In *Escherichia coli*, the fidelity of DNA replication is increased 100-fold by postreplication, methyl-directed DNA repair (10, 49, 52, 63). The misincorporated base on the newly replicated strand is distinguished from the correct base on the parent strand through the transient undermethylation of the daughter strand at 5′-GATC-3′ sequences, which are subsequently methylated by the *dam* gene product (16, 23, 45). The repair efficiency of specific base pair mismatches varies considerably. Transition mismatches are repaired most efficiently, while some transversion mismatches are repaired relatively poorly or not at all by methyl-directed mismatch repair (12, 30, 61). In addition to Dam methylation, the products of the *mutH*, *mutL*, *mutS*, and *uvrD* genes are required for methyl-directed mismatch repair both in vivo (2, 12, 17, 30, 43, 51) and in vitro (21, 36, 41, 43, 62, 64). By using an in vitro methyl-directed mismatch repair assay, the MutH, MutS, and MutL proteins of *E. coli* have been purified in a biologically active form (21, 62, 64). Both in vivo (37) and in vitro (64) studies suggest that strand discrimination occurs through the MutH-dependent nicking of the unmethylated strand at hemimethylated 5′-GATC-3′ sites. MutS has been shown to bind base pair mismatches (28, 61, 62) and to form a complex in the presence of ATP with MutL and DNA containing a mismatch (21). A putative nucleotide-binding site has been identified from the sequence of the *Salmonella typhimurium mutS* gene (22).

Streisinger and colleagues (59, 60) have proposed that frameshift mutations result from strand slippage during DNA replication through a run of identical bases, and consistent with this hypothesis, these sites are hot spots for deletion and insertion mutations (33, 56). Transfection experiments with heteroduplex DNA containing either 1 (13) or 10 (15) unpaired bases indicated that frameshift mutations are prevented by the methyl-directed mismatch repair pathway. The repair of heteroduplex substrates containing base additions, which crystallographic data indicate are radically different from substrates containing mismatched base pairs (4, 5, 27, 38, 48), has not yet been examined in vitro. Thus, we used the in vitro DNA mismatch repair assay (43) and a series of M13mp9 heteroduplex DNA substrates containing 1, 2, or 3 unpaired bases to study the repair efficiency of deletions and insertions. We demonstrate here that the methyl-directed mismatch repair pathway corrects heteroduplexes containing 1, 2, or 3 unpaired bases as efficiently as the G · T mismatch.

MATERIALS AND METHODS

Bacterial strains and bacteriophage. The bacterial strains CSH39 (47), RH411 (*mutH34*) (25), and RH721 (Δ*mutH1*) (24) have been described previously. CSH39 *mutS104*::Tn*5* and CSH39 *mutL103*::Tn*5* mutants were obtained from R. Hoess (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.). JM103 Δ*dam-16* was constructed by P1 transduction of Δ*dam-16* from GM3819 (45) (obtained from M. Marinus, University of Massachusetts Medical School, Worcester, Mass.) into JM103 (46). Transformants were selected by their resistance to kanamycin and sensitivity to 2-aminopurine (17, 18). The lack of DNA methylation was confirmed by digestion of genomic DNA with *Dpn*I and *Mbo*I, which cleave Dam-methylated and unmethylated DNAs, respectively (16, 34). Bacteriophage M13mp9 (65) was obtained from K. Abremski (du Pont).

Unmethylated phage and replicative-form (RF) DNAs were isolated from JM103 Δ*dam-16*, while methylated phage and RF DNAs were isolated from JM103 *dam*$^+$. Double-stranded, covalently closed RF DNA was isolated from infected cells and purified by centrifugation in CsCl-ethidium bromide density gradients by standard procedures (44). The methylated M13mp9 RF DNA was judged to be completely methylated based on digestion with *Dpn*I and *Mbo*I restriction endonucleases. Single-stranded phage DNA was isolated from purified phage. Phage were precipitated from infected cell supernatants with polyethylene glycol and purified by CsCl equilibrium density gradient centrifugation (54). Phage DNA was isolated by extensive treatment with proteinase K at 55°C in the presence of 1% sodium dodecyl sulfate, phenol extraction, and ethanol precipitation.

Materials. Proteinase K, T4 DNA ligase, and *E. coli* DNA polymerase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). *dam* methylase was purchased from New England BioLabs, Inc. (Beverly, Mass.). All restriction endonucleases were purchased from Bethesda Research Laboratories (Gaithersburg, Md.), except for

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*Hin*cII and *Hpa*I, which were purchased from New England BioLabs. Nitrocellulose type RS was the generous gift of Hercules Powder Co. (Wilmington, Del.).

Cell extracts. Cell extracts were prepared essentially as described previously (43), but on a micro level without the need for ultracentrifugation. Bacterial cultures were grown at 37°C to an A_{600} of 1.0 in 500 ml of LB broth (47) supplemented with 0.1% glucose. Cells were harvested by centrifugation, washed with 10 ml of cold 10 mM Tris hydrochloride (pH 7.6)–150 mM NaCl, and suspended in 1 ml of 50 mM Tris hydrochloride (pH 7.6)–10% sucrose. Cells were stored in 0.4-ml aliquots under argon at −80°C.

After the 0.4-ml aliquots were thawed at 4°C for 20 min, cells were lysed by the addition of 33 μl of 2 M KCl–1 μl of 1 M β-mercaptoethanol–5 μl of 10 mg of lysozyme per ml and incubation on ice for 45 min. Following a 1-min incubation at 37°C, the lysate was chilled to 0°C and centrifuged in a microcentrifuge at 12,000 × *g* for 15 min at 4°C. The lysis step was critical in the preparation of these extracts, and the optimum amount of lysozyme was determined empirically, yielding 0.15 to 0.2 ml of supernatant at this point. The supernatant was treated with 3 volumes of saturated $(NH_4)_2SO_4$ (adjusted to pH 7.2 with Tris base) for 30 min on ice and centrifuged at 12,000 × *g* for 15 min at 4°C. Pellets were gently suspended with 50 μl of 25 mM potassium-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.6)–100 mM KCl–0.1 mM sodium EDTA–5 mM β-mercaptoethanol and dialyzed against 200 ml of this buffer for 2 h at 4°C. Dialysis was performed on a membrane (type VS; Millipore Corp., Bedford, Mass.) in an atmosphere of argon. This crude extract (150 μl) contained 10 to 20 mg of protein per ml, as determined by a protein assay (Bio-Rad Laboratories, Richmond, Calif.), and was stored at −80°C in 25-μl aliquots under argon. Extracts prepared by this protocol have been stable for at least 6 months. Typically, 15 μg of wild-type extract fully repaired 240 ng of heteroduplex DNA in 40 min (see Fig. 7A).

Preparation of M13mp9 mutant phage. Synthetic 30-base oligonucleotides complementary to the multiple cloning region of M13mp9 phage DNA were prepared by A. Yeung (Fox Chase Cancer Center, Philadelphia, Pa.). Each of these oligonucleotides carried 1, 2, or 3 additional bases, disrupting the recognition sequence of unique restriction endonuclease sites within the multiple cloning region of M13mp9 and creating a new, unique restriction endonuclease sequence (see Table 1). Mutant M13mp9 phage containing 1-, 2-, or 3-base frameshift mutations were prepared by using oligonucleotide-directed mutagenesis (32). After hybridizing the oligonucleotides to M13mp9 phage DNA, fully duplex DNA was synthesized in a simultaneous DNA polymerase-DNA ligase reaction. DNA products were transformed into competent JM103 and mutant phage were selected as white plaques on isopropyl-β-D-thiogalactopyranoside–5-bromo-4-chloro-3-indolyl-β-D-galactoside agar plates. The identity of each mutant phage was confirmed by restriction analysis of RF DNA.

A mutant M13mp9 phage containing a single point mutation was constructed by methoxyamine mutagenesis (29). M13mp9 duplex DNA was linearized with *Sal*I, treated with methoxyamine, and religated. DNA was transformed into JM103, and mutant phage were selected as described above. This M13mp9 mutant contained a C-to-T base substitution within the *Sal*I recognition sequence, resulting in an opal mutation in codon 7 of the multiple cloning sequence (see Table 1). The mutant phage RF DNA was resistant to cutting with *Sal*I and *Acc*I but was still sensitive to *Hin*cII.

Heteroduplex DNA substrates. Heteroduplex DNA substrates were constructed essentially under the conditions of Kramer et al. (30). Methylated M13mp9 RF DNA (24 μg) was linearized with *Bgl*II and denatured in 0.3 ml of 10 mM Tris hydrochloride (pH 7.6)–2 mM sodium EDTA by heating at 100°C for 5 min and chilling to 0°C. The denatured DNA was hybridized to 100 μg of unmethylated M13mp9 mutant phage DNA in 0.47 ml of 10 mM Tris hydrochloride (pH 7.2)–2 mM sodium EDTA–0.15 M NaCl at 64°C for 1 h and allowed to reach room temperature slowly (2 h). Following 10 min on ice, the DNA was adjusted to 2.0 ml in 20 mM Tris hydrochloride (pH 7.2)–2 mM sodium EDTA–300 mM NaCl and applied to a 2-ml nitrocellulose column to remove unhybridized single-stranded DNA exactly as described (3), previously except that the column was equilibrated in 20 mM Tris hydrochloride (pH 7.2)–2 mM sodium EDTA–300 mM NaCl. The column eluant, containing nicked circular heteroduplex DNA, was precipitated with ethanol and used directly for in vitro DNA repair assays.

In vitro DNA repair assays. In vitro DNA repair assay conditions were those described previously (43), except that the reactions (10 μl) contained 120 ng of DNA and 15 to 30 μg of crude extract and were stopped after 1 h at 37°C by the addition of 90 μl of 10 mM Tris hydrochloride (pH 7.6)–25 mM sodium EDTA–1.2% *N*-lauroylsarcosine and proteinase K to 250 μg/ml. After incubation at 55°C for 1 h, DNA was extracted twice with an equal volume of phenol, precipitated with ethanol, and suspended in 10 μl of 5 mM Tris hydrochloride (pH 8.0)–1 mM sodium EDTA. Samples were analyzed for DNA repair by the addition of 1 μl of 500 mM Tris hydrochloride (pH 8.0)–100 mM $MgCl_2$–500 mM NaCl and 10 U of the appropriate restriction enzyme, as indicated in the figure legends, and incubated at 37°C for 30 min. Following electrophoresis at 5 mA/cm to resolve nicked circular DNA (40) on 0.8% (wt/vol) agarose gels, digestion products were visualized by staining the gels with ethidium bromide and photographed. Kinetic assays were performed with 240 ng of heteroduplex DNA per assay.

Purification of MutH and MutS. The cloning, identification, and sequence of the *mutH* gene has been described previously (19, 20). MutH was purified by column chromatography over DEAE-Sephacel, Affi-Gel Blue, Phenyl-Sepharose CL-4B, and Sephadex G-75 (manuscript in preparation). The specific activity (64) of MutH was 50,000 U/mg. The *mutS* gene was cloned on a 3.8-kilobase *Bgl*II fragment (62) into pUC18. Recombinant plasmids were screened by using a probe isolated from a Tn*5* insertion into the *mutS* gene of *E. coli*. Potential *mutS* clones were then screened for the complementation of the *mutS* phenotypes; they restored the spontaneous mutation frequency of *mutS* strains to wild-type levels and reverted the 2-aminopurine resistance of *dam mutS* double mutants to the sensitivity of that found with *dam mut*$^+$ strains (data not shown). MutS was purified essentially as described previously (62), with a specific activity of 1,050 U/mg (62).

RESULTS

Frameshift heteroduplex DNA substrates. The M13mp9 genome contains seven GATC sequences which are targets for Dam methylation (65). The level of methylation is more difficult to determine for single-stranded DNA than for double-stranded DNA, which can be analyzed by digestion with *Dpn*I and *Mbo*I (16, 34). Hence, the unmethylated strand was prepared from the purified phage and the methylated strand was prepared from the RF DNA. Hemimeth-

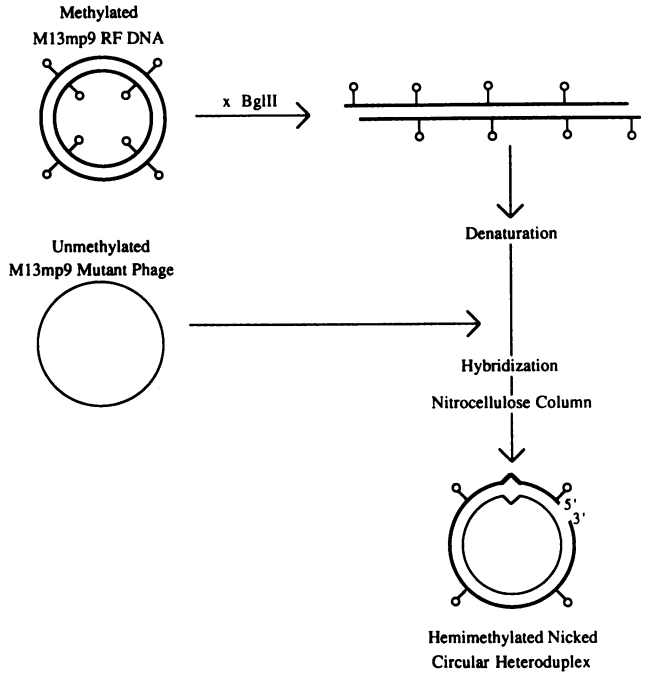

FIG. 1. Preparation of the hemimethylated +1 frameshift heteroduplex substrate. Thick lines represent RF DNA, and thin lines indicate phage DNA. The open circles indicate modification by the Dam methylase.

ylated heteroduplex substrates were prepared by hybridizing the linearized, denatured, wild-type M13mp9 RF DNA to an excess of single-stranded M13mp9 mutant phage DNA (Fig. 1). After separation of the nicked circular heteroduplex DNA from unhybridized single-stranded DNA by chromatography over nitrocellulose (6), there was very little contamination of the nicked circular substrate with rehybridized linear double-stranded DNA (see lane 2 in Fig. 2 and in Fig. 6A and B). The frameshift heteroduplexes contained 1-, 2-, or 3-base additions in the multiple cloning site of M13mp9 DNA on the phage strand, creating the recognition sequence of a new, unique restriction endonuclease site (Table 1). Similar substrates containing single-base-pair mismatches within unique restriction enzyme sites were previously used to study methyl-directed DNA mismatch repair in vitro (1, 21, 36, 41–43, 61, 62, 64). Repair of the mismatch was detected by the sensitivity of the repaired DNA to the appropriate restriction enzyme. In the case of the three frameshift heteroduplex substrates, none was susceptible to double-strand cutting by the indicator restriction enzymes without first undergoing in vitro DNA repair (see below).

Methyl-directed repair of single-base frameshifts in vitro. To determine whether base insertions were corrected via the methyl-directed mismatch repair pathway in vitro, a hemimethylated heteroduplex containing a single unpaired base was tested for repair in a cell extract from wild-type *E. coli*. The hemimethylated +1 frameshift heteroduplex contained an additional unpaired adenine on the unmethylated strand, creating an *Nhe*I-sensitive site (Table 1). As stated above, the presence of the unpaired base rendered this nicked circular heteroduplex substrate (Fig. 2, lane 2) resistant to cutting by either *Pst*I or *Nhe*I (Fig. 2, lanes 4 and 5, respectively). The position of the linear DNA is shown by the digestion with *Hin*dIII (Fig. 2, lane 3), an enzyme that cleaves distal to the heteroduplex site. After incubation with the wild-type extract (Fig. 2, lanes 6 to 8), the DNA repair

TABLE 1. M13mp9 hemimethylated heteroduplex substrates

Substrate	Phage[a]	Heteroduplex site[b]	Marker
G · T mismatch	M13mp9	c 3′–ACCAGCTGCCTA	*Sal*I
	M13mp9-OP-7	p 5′–TGGTTGACGGAT	
+1 frameshift	M13mp9	c 3′–CCGA CGTCCAG	*Pst*I
	M13mp9 FS1	p 5′–GGCTAGCAGGTC	*Nhe*I
−1 frameshift	M13mp9 FS1	c 3′–CCGATCGTCCAG	*Nhe*I
	M13mp9	p 5′–GGCT GCAGGTC	*Pst*I
+2 frameshift	M13mp9	c 3′–GGTTC GAACC	*Hin*dIII
	M13mp9 FS2	p 5′–CCAAGGCCTTGG	*Stu*I
+3 frameshift	M13mp9	c 3′–ACCA GCTGC	*Sal*I
	M13mp9 FS3	p 5′–TGGTTAACGACG	*Hpa*I

[a] OP-7, Opal mutation in the 7th codon within the multiple cloning site of the α-complementing portion of β-galactosidase; FS1, +1 frameshift; FS2, +2 frameshift; FS3, +3 frameshift.

[b] c, Methylated complementary strand; p, unmethylated phage strand. Relevant restriction endonuclease sequences are underlined. The 1-, 2-, or 3-base insertions and the G · T base substitution are shown in bold type.

products (Fig. 2, lane 6) were more sensitive to digestion with *Pst*I (Fig. 2, lane 7) than *Nhe*I (Fig. 2, lane 8), indicating that the heteroduplex is preferentially repaired by removal of the extra unpaired base on the unmethylated strand. The low but reproducible level of repair, which occurred on the methylated strand (*Nhe*I-sensitive DNA; Fig. 2, lane 8) was most likely the result of nick translation since it was independent of *mutH*, *mutL*, and *mutS* (see below). Conversion of the nicked circular DNA to the linear form was not detected without the addition of extract and the appropriate restriction enzyme (Fig. 2, lanes 2, 6, and 7).

The results presented above are consistent with either methyl-directed repair or preferential removal of the extra unpaired adenine. To distinguish between these two possibilities, a hemimethylated −1 frameshift heteroduplex was

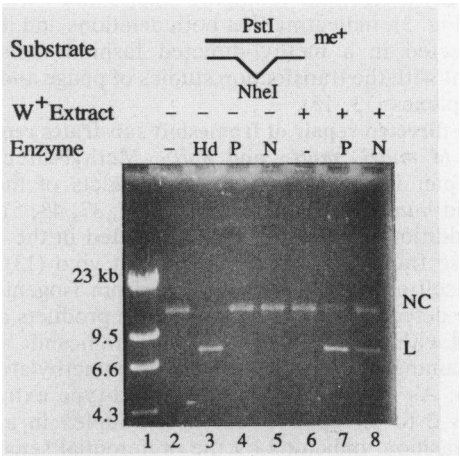

FIG. 2. In vitro methyl-directed repair of a single-base insertion. The hemimethylated +1 frameshift heteroduplex (lane 2) was linearized with *Hin*dIII (lane 3), but was resistant to *Pst*I (lane 4) and *Nhe*I (lane 5). After incubation with a wild-type extract (lanes 6 to 8), DNA repair products (lane 6) were analyzed by digestion with *Pst*I (lane 7) or *Nhe*I (lane 8). Lane 1, Lambda phage DNA digested with *Hin*dIII. Abbreviations: Hd, *Hin*dIII; P, *Pst*I; N, *Nhe*I; NC, nicked circular DNA; L, linear DNA; W+, wild type; kb, kilobase; me+, methylated DNA. The thick line represents the complementary DNA strand, and the thin line indicates the phage DNA strand.

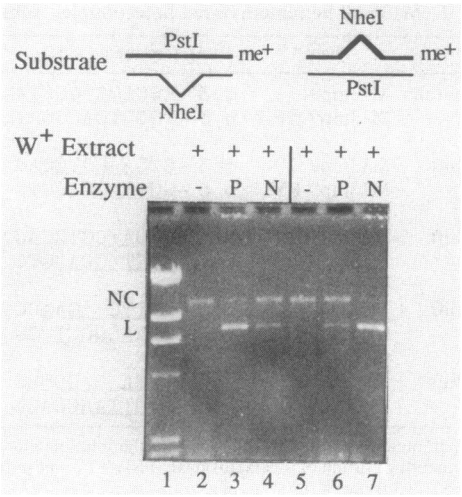

FIG. 3. In vitro methyl-directed repair of single-base insertion or deletion. The hemimethylated +1 frameshift heteroduplex (lanes 2 to 4) or −1 frameshift heteroduplex (lanes 5 to 7) was incubated with a wild-type cell extract. DNA repair products (lanes 2 and 5) were analyzed by digestion with *Pst*I (lanes 3 and 6) or *Nhe*I (lanes 4 and 7). Lane 1, Lambda phage DNA digested with *Hin*dIII. Abbreviations are as defined in the legend to Fig. 2.

constructed by using unmethylated wild-type M13mp9 phage DNA and methylated M13mp9 +1 frameshift RF DNA (Table 1). This heteroduplex contained an additional unpaired thymine on the methylated strand. Methyl-directed repair of this substrate would lead to a *Nhe*I-sensitive product, while preferential removal of the extra base would result in a *Pst*I-sensitive product. Comparison of the in vitro repair of the +1 and −1 frameshift heteroduplexes (with the former containing an extra base on the unmethylated strand and the latter containing an extra base on the methylated strand) is shown in Fig. 3. The results demonstrate that repair of both heteroduplex sites is preferentially directed to the unmethylated strand (compare lanes 3 and 4 with lanes 6 and 7 in Fig. 3), indicating that both deletions and insertions are corrected in a methyl-directed fashion, which is in agreement with the transfection studies of phage and plasmid heteroduplexes (13, 15).

Methyl-directed repair of frameshift substrates requires the products of *mutH*, *mutL*, and *mutS*. Methyl-directed mismatch repair is dependent on the products of the *mutH*, *mutL*, and *mutS* genes (2, 12, 17, 21, 30, 37, 43, 51, 61, 62, 64). In addition, *mutL* has been implicated in the repair of single-base frameshift heteroduplexes in vivo (13). Consequently, cell extracts were prepared from isogenic strains that were defective in each of these gene products and were incubated with the hemimethylated +1 frameshift heteroduplex containing an extra adenine on the unmethylated strand (Fig. 4A). As described above, the wild-type extract (Fig. 4A, lanes 2 to 4) repaired this heteroduplex in a methyl-directed fashion, indicated by the preferential sensitivity of the repair products to *Pst*I (compare lane 3 and 4 in Fig. 4A). However, after incubation of the +1 frameshift heteroduplex with cell extracts prepared from strains carrying *mutH* (Fig. 4A, lanes 5 to 7), *mutL* (Fig. 4A, lanes 8 to 10), and *mutS* (Fig. 4A, lanes 11 to 13), the DNA remained resistant to *Pst*I (Fig. 4A, lanes 6, 9, and 12), indicating the loss of methyl-directed repair in these mutant extracts, as expected. Since only a low level of *Nhe*I-sensitive repair products was observed (Fig. 4A, lanes 7, 10, and 13), very little of the

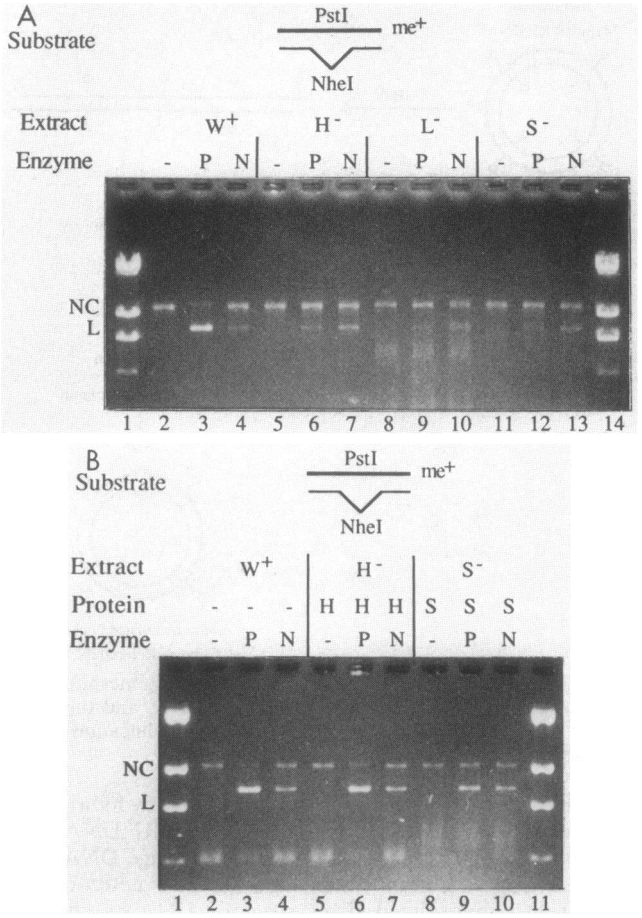

FIG. 4. In vitro repair of single-base insertions in *mutH*, *mutL*, and *mutS* extracts. (A) The hemimethylated +1 frameshift heteroduplex was incubated with cell extracts prepared from strains CSH39 wild type (W^+; lanes 2 to 4), CSH39 *mutH34* (H^-; lanes 5 to 7), CSH39 *mutL103*::Tn*5* (L^-; lanes 8 to 10), or CSH39 *mutS104*::Tn*5* (S^-; lanes 11 to 13). DNA repair products (lanes 2, 5, 8, and 11) were analyzed by digestion with *Pst*I (lanes 3, 6, 9, and 12) or *Nhe*I (lanes 4, 7, 10, and 13). (B) Heteroduplexes were incubated as described above for panel A in CSH39 wild-type (lanes 2 to 4), RH721 (Δ*mutH1*) (lanes 5 to 7), or CSH39 *mutS*::Tn*5* (lanes 8 to 10) cell extracts, except that 0.5 U of purified MutH (H; lanes 5 to 7) or 0.02 U of purified MutS (S; lanes 8 to 10) was added to the incubations. DNA repair products (lanes 2, 5, and 8) were analyzed by digestion with *Pst*I (lanes 3, 6, and 9) or *Nhe*I (lanes 4, 7, and 10). Lambda phage DNA was digested with *Hin*dIII in lanes 1 and 14 of panel A and lanes 1 and 11 of panel B. Abbreviations are as defined in the legend to Fig. 2.

hemimethylated +1 frameshift heteroduplex was repaired on either strand in *mutH*, *mutL*, and *mutS* extracts. In addition, essentially the same level of *Nhe*I-sensitive DNA was observed following incubation with the wild-type (Fig. 4A, lane 4) and the mutant (Fig. 4A, lanes 7, 10, and 13) extracts, suggesting that this repair on the methylated strand is the result of nick translation across the heteroduplex site (see below). When the *mutH* or *mutS* extracts were supplemented with purified MutH (Fig. 4B, lanes 5 to 7) or MutS (Fig. 4B, lanes 8 to 10) proteins, respectively, repair of the +1 frameshift heteroduplex occurred in a methyl-directed manner at near wild-type levels, as indicated by the *Pst*I-sensitive DNA repair products (Fig. 4B, lanes 3, 6, and 9). In addition, methyl-directed repair could be restored to *mutL*

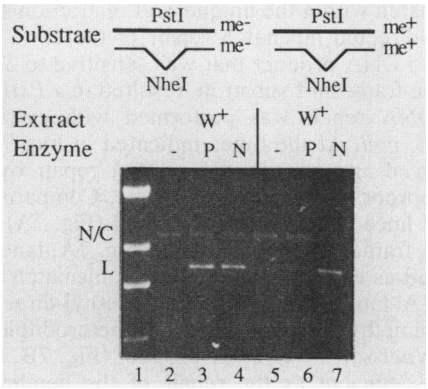

FIG. 5. In vitro repair of unmethylated or fully methylated single-base insertion heteroduplexes. The +1 frameshift heteroduplexes, either unmethylated (lanes 2 to 4) or fully methylated (lanes 5 to 7), were incubated with a wild-type cell extract. DNA repair products (lanes 2 and 5) were analyzed by digestion with *Pst*I (lanes 3 and 6) or *Nhe*I (lanes 4 and 7). Lane 1, Lambda phage DNA digested with *Hin*dIII. me^-, Unmethylated DNA; me^+, methylated DNA. Other abbreviations are as defined in the legend to Fig. 2.

extracts by the addition of partially purified preparations of MutL protein (data not shown). Since *E. coli* DNA ligase was present in these extracts, the DNA migrating at approximately 4.3 kilobases was the result of supercoiling of the nicked circular substrate during the mismatch correction assay. The extent of supercoiling varied from extract to extract.

Repair of fully unmethylated or fully methylated M13mp9 +1 frameshift hybrids. Previous studies have demonstrated that unmethylated heteroduplexes containing a mismatch are repaired without strand bias, while the repair of fully methylated substrates is greatly reduced (12, 15, 30, 36, 41, 43, 51). Thus, to further establish that the repair of the +1 frameshift heteroduplex was the product of methyl-directed DNA mismatch repair, we tested substrates that were either fully methylated or completely unmethylated. Fully unmethylated +1 frameshift substrates were constructed from mutant phage DNA and wild-type RF DNA isolated from a strain with a *dam* deletion. Fully methylated substrates were constructed from DNA isolated from a dam^+ host, and the heteroduplex substrate was subjected to in vitro methylation with purified Dam methylase. The methylation state of each of the hybrids was checked by restriction enzyme analysis with *Dpn*I and *Mbo*I (data not shown). Following incubation of the unmethylated +1 frameshift heteroduplex with the wild-type extract (Fig. 5, lanes 2 to 4), the DNA repair products were equally sensitive to *Pst*I and *Nhe*I (Fig. 5, lanes 3 and 4), indicating that this substrate is repaired without strand bias. By contrast, the fully methylated +1 frameshift (Fig. 5, lanes 5 to 7) was inefficiently repaired on either strand since there was a very low level of sensitivity to *Pst*I (Fig. 5, lane 6) and since equal levels of *Nhe*I-sensitive DNA products were observed following incubation in a wild-type (Fig. 5, lane 7) and a *mutS* (data not shown) extract. These results are consistent with those of previous studies of methyl-directed repair with either unmethylated or fully methylated mismatch heteroduplexes.

MutH has been proposed to direct the mismatch repair complex by nicking the unmethylated strand of hemimethylated DNA at 5′-GATC-3′ sites (35, 37, 64). Since the M13mp9 RF DNA used to prepare our substrates was linearized with the restriction enzyme *Bgl*II, the sequence at the site of the nick on the hemimethylated heteroduplex was also 5′-GATC-3′. Thus, the nick on the methylated strand of our substrates could mimic a MutH cleavage site (35, 64) and direct repair to the methylated strand, creating a *Nhe*I-sensitive site (Fig. 1). Although the low levels of sensitivity to the unmethylated strand marker endonuclease (Fig. 3, lanes 4 and 6; Fig. 4A, lanes 4 and 7) are consistent with the fact that some nick-directed DNA mismatch repair occurred in these extracts, this was not the case since approximately the same level of sensitivity to *Nhe*I was seen in extracts prepared from strains carrying either *mutS* or *mutL* (Fig. 4A, lanes 10 and 13). The sensitivity to the unmethylated strand marker endonuclease was most likely due to nick translation across the heteroduplex site because equal levels were observed in both wild-type and *mut* mutant extracts and because this sensitivity was the same for strands containing an insertion or a deletion (Fig. 3, lanes 4 and 6). The level of nick translation was low due to the rapid ligation of the DNA substrate in these crude extracts (data not shown) since the reaction mixtures contain NAD, the cofactor for *E. coli* DNA ligase (50). The level of nicked translated DNA was never greater than 20% of the total DNA in each assay based on densitometric analysis. Although the existence of an alternate repair pathway (e.g., *recF* dependent [14, 15]) for the repair of the heteroduplex site cannot be excluded, the low, but detectable, level of repair seen on the phage strand of the fully methylated substrate (Fig. 5, lane 6) suggested that this alternate repair pathway does not contribute significantly to the repair of frameshift mutations in this system.

Methyl-directed repair of 2- and 3-base frameshift substrates. Previous in vivo experiments examining the repair of frameshift heteroduplexes by transfection assays have only been performed with either single (13) or large (11, 15, 31) frameshift heteroduplexes. Consequently, heteroduplex substrates containing 2 or 3 extra unpaired bases were constructed and tested for in vitro methyl-directed repair. The M13mp9 +2 frameshift mutant phage used to prepare the +2 frameshift heteroduplex (Table 1) was constructed by inserting GC within the *Hin*dIII site of M13mp9, creating a *Stu*I restriction site. The hemimethylated +2 frameshift heteroduplex was resistant to digestion with either *Hin*dIII or *Stu*I (Fig. 6A, lane 2). Following incubation with the wild-type extract (Fig. 6A, lanes 3 to 5), the DNA repair products were much more sensitive to *Hin*dIII (compare lanes 4 and 5 in Fig. 6A), indicating preferential repair on the unmethylated strand. However, in a *mutS* extract (Fig. 6A, lanes 6 to 8), methyl-directed repair was abolished since essentially no *Hin*dIII-sensitive DNA was observed (Fig. 6A, lane 7). The equally low levels of *Stu*I-sensitive DNA following incubation with either wild-type (Fig. 6A, lane 5) or *mutS* (Fig. 6A, lane 8) extract were most likely due to nick translation and not DNA repair.

A +3 frameshift heteroduplex (Table 1) was prepared with mutant M13mp9 phage DNA containing a 3-base insertion (TAA) within the *Sal*I sequence creating a *Hpa*I restriction sequence. The hemimethylated +3 frameshift heteroduplex was resistant to both *Sal*I and *Hpa*I (Fig. 6B, lane 2). Analysis of the DNA repair products after incubation with the wild-type extract (Fig. 6B, lanes 3 to 5) demonstrated that repair occurred in a methyl-directed manner, since the repair products were predominantly sensitive to *Sal*I (compare lane 4 to 5 in Fig. 6B). This repair was MutS dependent (Fig. 6B, lanes 6 to 8) since essentially none of the DNA was *Sal*I sensitive after incubation with the *mutS* extract (Fig. 6B, lane 7). As with the 1- and 2-base frameshift heteroduplexes, the low level of sensitivity to the unmethylated

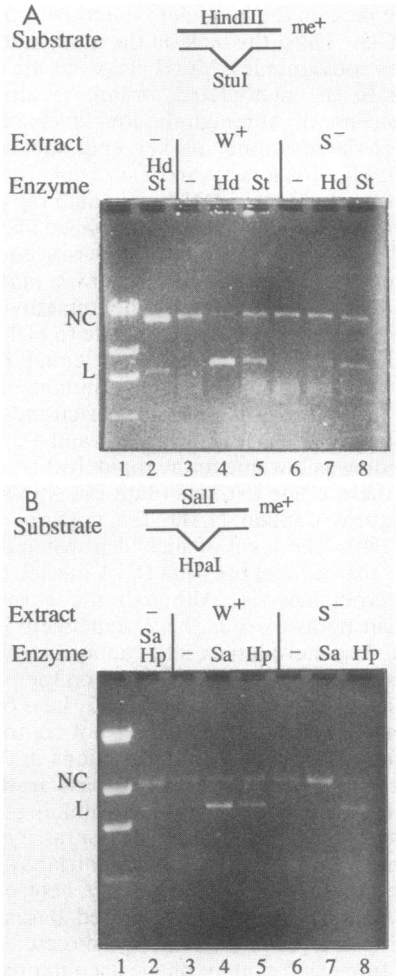

FIG. 6. In vitro repair of 2- and 3-base insertion heteroduplexes in wild-type and *mutS* extracts. (A) The hemimethylated +2 frameshift heteroduplex was incubated with a wild-type (lanes 3 to 5) or *mutS* (lanes 6 to 8) cell extract. DNA repair products (lanes 3 and 6) were analyzed by digestion with *Hin*dIII (lanes 4 and 7) or *Stu*I (lanes 5 and 8). The +2 frameshift heteroduplex was resistant to cutting by both *Hin*dIII and *Stu*I (lane 2). (B) The hemimethylated +3 heteroduplex was incubated with a wild-type (lanes 3 to 5) or *mutS* (lanes 6 to 8) cell extract. DNA repair products (lanes 3 and 6) were analyzed by digestion with *Sal*I (lanes 4 and 7) or *Hpa*I (lanes 5 and 8). The +3 frameshift heteroduplex was resistant to cutting by both *Sal*I and *Hpa*I (lane 2). Lanes 1, Lambda phage DNA digested with *Hin*dIII. Hp, *Hpa*I; Sa, *Sal*I; St, *Stu*I. Other abbreviations are as defined in the legend to Fig. 2.

strand marker endonuclease (*Hpa*I) after incubation with both the wild-type and *mutS* extracts (Fig. 6B, lanes 5 and 8) was probably the result of nick translation. These results suggest that 2- and 3-base frameshift mutations are also prevented in vivo by the methyl-directed MutHLS repair pathway.

Small frameshifts are repaired as efficiently as a G · T DNA mismatch. The rates of in vitro methyl-directed mismatch repair varied substantially between the various mismatched base pairs, with G · T mismatches being repaired most efficiently (61). Since in vivo studies have also suggested that the G · T mismatch is the most efficient target for methyl-directed repair (12, 30), repair of the hemimethylated +1 frameshift heteroduplex was compared with the repair of a similar hemimethylated M13mp9 heteroduplex containing a G · T mismatch within the unique *Sal*I restriction site (Table 1). Methyl-directed mismatch repair of the G · T substrate resulted in a DNA product that was sensitive to *Sal*I, while repair of the frameshift substrate resulted in a *Pst*I-sensitive product. DNA repair was performed with extracts from wild-type *E. coli*. At the times indicated in Fig. 7, aliquots were removed and examined for DNA repair by analysis with the appropriate restriction enzyme. Comparison of the amounts of linear DNA product formed (Fig. 7A) indicated that the +1 frameshift heteroduplex (Fig. 7A, lanes 8 to 13) was repaired as efficiently as the G · T mismatch heteroduplex (Fig. 7A, lanes 2 to 7). Similarly, methyl-directed repair of the hemimethylated −1 frameshift heteroduplex, which yielded a *Nhe*I-sensitive repair product (Fig. 7B, lanes 8 to 13), was as efficient as the repair of the hemimethylated G · T heteroduplex (Fig. 7B, lanes 2 to 7). Finally, since a single-base insertion or deletion was corrected as efficiently as a G · T mismatch (Fig. 7A and B), repair of the hemimethylated +3 frameshift heteroduplex was also compared with that of the hemimethylated G · T mismatch heteroduplex. Since methyl-directed repair of both of these heteroduplexes resulted in a *Sal*I-sensitive repair product, DNA repair products were analyzed by digestion with *Sal*I following incubation with the wild-type extract for the times indicated in Fig. 7C. The repair of the hemimethylated +3 heteroduplex (Fig. 7C, lanes 8 to 13), as indicated by the amount of linear DNA, was as efficient as that of the hemimethylated G · T mismatch substrate (Fig. 7C, lanes 2 to 7). The +2 frameshift heteroduplex was also repaired as efficiently as the G · T mismatch heteroduplex (data not shown). Thus, the methyl-directed MutHLS repair pathway corrects small base insertions or deletions in vitro as efficiently as the G · T base mismatch.

DISCUSSION

The specificity of the MutHLS repair pathway to correct small base insertions and deletions was studied by using an in vitro DNA mismatch repair assay. Previously, this in vitro assay demonstrated that the MutHLS pathway repairs specific base mismatches with different efficiencies, confirming transfection experiments with mismatch heteroduplex DNA. The G · T mismatch is corrected most efficiently, while the A · C, C · T, A · A, T · T, and G · G mispairs are corrected at rates of 40 to 80% of the G · T mispair, and the C · C mispair is repaired very poorly (12, 30, 61). Transfection experiments have demonstrated methyl-directed, *mutL*-dependent repair of heteroduplex phage DNA containing single-base insertions or deletions (13). However, similar studies have suggested that MutHLS-dependent and MutHLS-independent pathways are involved in the repair of larger insertions and deletions (11, 15, 31). As noted previously (12, 61), transfection studies score the progeny of heteroduplex phage, and the results of these experiments depend not only on DNA repair but also on the rate at which DNA replication resolves the heteroduplex and on strand bias during DNA replication. Thus, it remains unclear from results of the in vivo studies how efficiently the MutHLS pathway repaired base insertions and deletions, whether there was a limit to the size of the insertion or deletion that could be repaired, and whether other pathways were involved in the repair of these types of replication errors.

The spontaneous mutation spectra in strains carrying *mutH*, *mutL*, *mutS*, and *dam* are essentially identical, and transitions make up the major class of mutations in these mismatch repair-deficient strains (8, 9, 39, 53, 56). If the

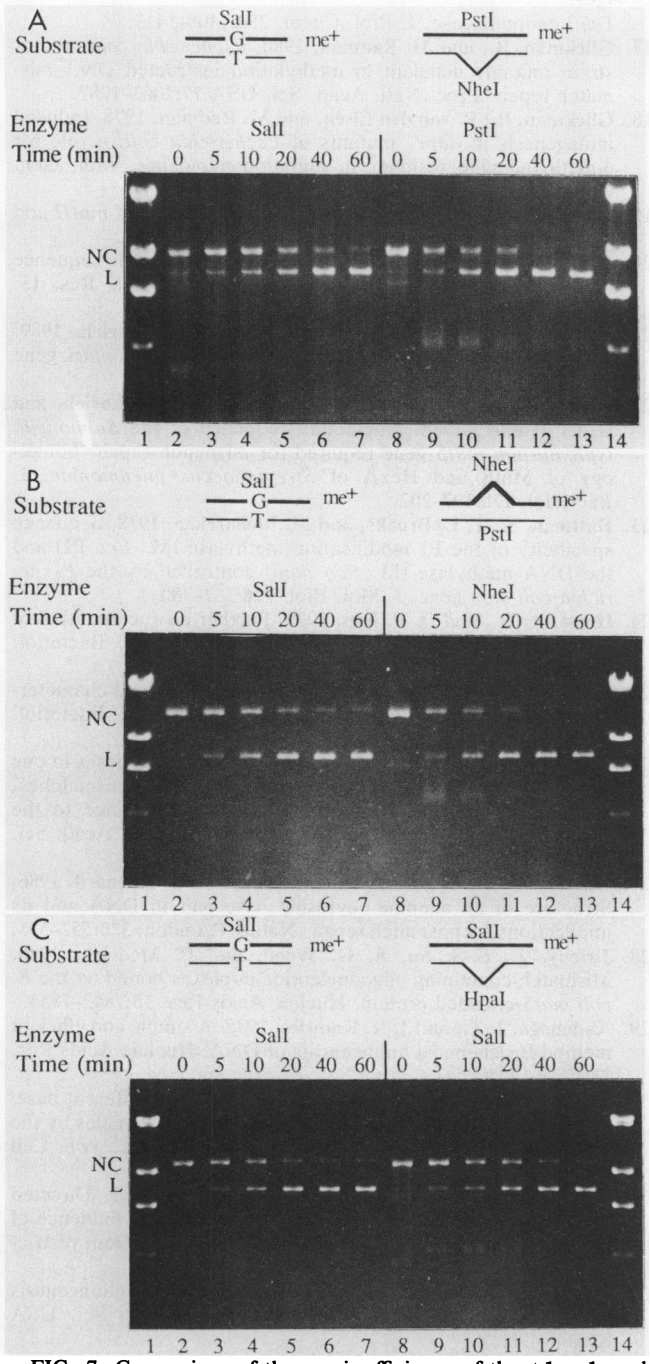

FIG. 7. Comparison of the repair efficiency of the +1, −1, and +3 frameshift heteroduplexes to a G · T base mismatch heteroduplex. (A) The hemimethylated G · T mismatch heteroduplex (lanes 2 to 7) and the +1 frameshift heteroduplex (lanes 8 to 13) were incubated with a wild-type cell extract. At the indicated times, aliquots were extracted and the DNA repair products were analyzed by digestion with *Sal*I (lanes 2 to 7) or *Pst*I (lanes 8 to 13). The zero time point actually reflects a 15- to 30-s time point since the cell extract was added to the prewarmed reaction mixture. (B) Incubation was as described above for panel A, except that the hemimethylated −1 frameshift heteroduplex (lanes 8 to 13) DNA repair products were analyzed by digestion with *Nhe*I. (C) Incubation was as described above for panel A, except that the hemimethylated +3 frameshift heteroduplex (lanes 8 to 13) DNA repair products were analyzed by digestion with *Sal*I. Lanes 1 and 14, Lambda phage DNA digested with *Hin*dIII. Other abbreviations are as defined in the legend to Fig. 2.

spontaneous mutation spectrum accurately represents DNA polymerase errors, then these studies reflect the mismatch repair specificity of the MutHLS pathway. Although rare in wild-type *E. coli* (55), single-base deletion frameshifts make up 25% of the spectra of *lacI* gene mutations in strains carrying *mutH*, *mutL*, or *mutS* (56). However, in studies examining mutations in the P22 *mnt* gene, no single-base and only one 12-base deletion mutant was detected out of 47 mutants in a strain carrying *mutH* (53). Additionally, only 4 single-base frameshifts and two large deletions were detected out of 91 isolates from a strain carrying *dam* (8). These differences between the *lacI* and *mnt* genes in the frequency and types of frameshift mutations could be explained by the lack of frameshift hot spots in the *mnt* gene or by insufficient sample size.

The results presented in this report confirm and expand the earlier in vivo studies implicating the methyl-directed DNA repair pathway in the prevention of frameshift mutations (13, 15, 25, 56, 58). Hemimethylated heteroduplexes containing 1- to 3-base insertions or deletions are repaired in a methyl-directed fashion in extracts prepared from wild-type *E. coli* but not from *mutH*, *mutL*, or *mutS* mutants. The addition of purified MutH or MutS to extracts prepared from strains carrying, *mutH* or *mutS*, respectively, restores methyl-directed repair activity. Although our results demonstrate that 1- to 3-base insertions are repaired as efficiently as the G · T mismatch, the maximum size of the insertion or deletion that is efficiently repaired by the MutHLS pathway in vitro has not yet been determined.

The A · G mismatch is repaired inefficiently in vivo and in vitro by the MutHLS pathway (12, 30, 61). Recently, in vitro studies have identified a new repair pathway for this mismatch that does not require MutHLS (1, 7, 42, 57, 61). Although the results presented here demonstrate that the MutHLS pathway is the major repair mechanism for small insertions or deletions, the existence of an alternate repair pathway for larger regions of unpaired DNA cannot be excluded. A low but detectable level of repair occurred on the phage strand of the fully methylated substrate (Fig. 5, lane 6) that could be indicative of an alternate DNA repair mechanism (14, 15, 36). A *recF*-dependent pathway for the repair of frameshift heteroduplexes that is independent of the state of DNA methylation has been identified both in vivo (15) and in vitro (14). However, it has not been shown in vitro whether this alternate DNA repair pathway is directed by the frameshift error or is simply the result of random nicking of the DNA with concomitant nick translation. The low level of correction that occurred on the methylated strand of our hemimethylated substrates was probably due to nick translation since this repair (i) was not dependent on *mutH*, *mutL*, or *mutS* (Fig. 4A and B); (ii) did not preferentially repair insertions or deletions (Fig. 3); and (iii) occurred predominantly on the nicked strand of fully methylated heteroduplexes (Fig. 5).

X-ray crystallographic studies indicate that G · T (5) and A · C (27) mismatches form nearly perfect Watson-Crick base pairs, resulting in little, if any, distortion of the DNA helix. Similarly, duplex oligonucleotides containing a single-base insertion or deletion also result in minimal distortion of the overall helix but have the unpaired base looped out either into the minor groove or lying along the phosphodiester backbone (38, 48). More recent experiments have suggested that DNA containing an extra unpaired base is bent, while that with a mismatched base is not (26). Since the physical evidence suggests that mismatches and single-base insertions or deletions are very different structures, it is interest-

ing that both of these types of replication errors are efficiently corrected by the methyl-directed repair pathway. Our results demonstrate that MutS is required for the methyl-directed repair of single-base insertions or deletions in vitro, suggesting that MutS either binds as efficiently to potential frameshift mutations as it does to G · T mismatches or that this binding is not the rate-limiting step in methyl-directed DNA repair. Experiments are currently under way to answer this question.

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