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Specific A/G-to-C·G Mismatch Repair in *Salmonella typhimurium* LT2 Requires the *mutB* Gene Product

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An assay has been developed that permits analysis of repair of A/G mismatches to C·G base pairs in cell extracts of *Salmonella typhimurium* LT2. This A/G mismatch repair is independent of ATP, *dam* methylation, and *mutS* gene function. The gene product of *mutB* has been shown to be involved in the *dam*-independent pathway through the in vitro assay. Moreover, specific DNA-protein complexes and an endonuclease can be detected in *S. typhimurium* extracts by using DNA fragments containing an A/G mismatch. These activities are not observed with substrates which have a T/G mismatch or no mismatch. The *S. typhimurium* endonuclease, like the A/G endonuclease found in *Escherichia coli* (A-L. Lu and D.-Y. Chang, Cell 54:805–812, 1988), makes incisions at the first phosphodiester bond 3′ to and the the second phosphodiester bond 5′ to the dA of the A/G mismatch. No incision site was detected on the other DNA strand. Extracts prepared from *mutB* mutants cannot form A/G mismatch-specific DNA-protein complexes and do not contain the A/G endonuclease activity. Thus the A/G mismatch specific binding and nicking activities are probably involved in the A/G mismatch repair pathway. Preliminary analysis of the mutational spectrum of the *mutB* strain has indicated that this mutator allele causes an increase in C·G-to-A·T transversions without affecting the frequencies of other transversion or transition events. In addition, the *mutB* gene has been mapped to the 64-min region of the *S. typhimurium* chromosome. Together, this biochemical and genetic evidence suggests that the *mutB* gene product of *S. typhimurium* is the homolog of the *E. coli micA* (and/or *mutY*) gene product.

DNA base-pair mismatches may arise from spontaneous replication error, deamination of 5-methylcytosine, and homologous genetic recombination. Mismatch repair directed by *dam* methylation at d(GATC) sequences is believed to eliminate DNA replication errors in both *Escherichia coli* and *Salmonella typhimurium* (reviewed in references 9, 32, and 39). Repair is biased to the unmethylated newly synthesized DNA strand which bears the replication errors. Methyl-directed mismatch correction in *E. coli* requires DNA helicase II (the gene product of *uvrD*), single-stranded DNA-binding protein, exonuclease I, DNA polymerase III holoenzyme, and the products of the *mutH*, *mutL*, and *mutS* genes (4, 18, 19, 27, 33, 37, 41). Mutations of *S. typhimurium* corresponding to the *mutH*, *mutL*, *mutS*, and *uvrD* genes of *E. coli* have been isolated (44). Plasmids that contain the *mutH*, *mutL*, and *mutS* genes of *S. typhimurium* are able to complement the spontaneous mutator phenotype of the corresponding *E. coli* mutants (36), suggesting that mismatch repair proteins are functionally equivalent in *S. typhimurium* and *E. coli*.

Besides the *dam* methylation-dependent pathway, there are several *dam*-independent repair systems that act on heteroduplex DNA. In *E. coli*, very-short-patch repair (22, 24, 40) acts unidirectionally on a G/T mismatch to restore the G·C base pair at the second position within the sequence 5′-CC(A/T)GG and also within certain related sequences (23, 24). This system corrects G/T mispairs that arise by deamination of 5-methylcytosine in G·m^{5}C base pairs (17) and requires intact *mutL* and *mutS* genes but not *mutH* and *uvrD* genes (17, 39). Two low-efficiency methylation-independent pathways have been identified in *E. coli* by using plamid DNA heteroduplexes (12, 13). One pathway involves long excision tracts and does not require *mutH* or *mutL* function, but requires the *mutS* and *uvrD* gene products. The second pathway is dependent on the *recF* and *recJ* functions. In contrast to *dam*-directed and very-short-patch repair systems, repair by these two pathways can occur on either DNA strand. There is also evidence for a C/C mismatch repair system that is independent of *mutS*, *mutL*, and *dam* methylation (38).

Recently, another *dam*-independent repair pathway in *E. coli* was described (25, 38, 45). This repair system is specific for A/G mismatches (and A/C mismatches, as shown in vivo), is unidirectional with conversion of A/G mismatches to C·G base pairs, and is not controlled by host *mutH*, *mutL*, *mutS*, *uvrD*, *recF*, and *recJ* gene functions. However, this A/G mismatch correction system is dependent on the newly described *micA* and/or *mutY* gene products (2, 38; A-L. Lu, J.-J. Tsai, J. P. Radicella, and M. S. Fox, manuscript in preparation). *micA* and *mutY* strains are mutators, and *mutY* mutants exhibit a marked increase in C·G-to-A·T transversions (34, 38). From genetic mapping and functional analyses, *micA* and *mutY* mutations may be different alleles of a gene or mutations in closely linked genes; therefore, they are currently expressed as *micA* (and/or *mutY*). The *micA* (and/or *mutY*) dependence of A/G-specific mismatch repair strongly suggests a role for this repair system in the prevention of C·G-to-A·T transversions during DNA replication. The repair of A/G mismatches to C·G base pairs may also be involved in gene conversion within regions of heteroduplex DNA formed during genetic recombination. Two tightly associated activities, the specific binding and nicking of DNA fragments containing A/G mismatches, have been identified in *E. coli* cell extracts and are involved in the A/G mismatch repair pathway (26). The specific endonuclease simultaneously makes two incisions (only two nucleotides apart) near the mismatched site on the DNA strand which contains the dA of the A/G mismatch but has no detectable

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incision site on the opposing DNA strand. A nucleotide excision repair model has been proposed for the conversion of an A/G mismatch to a C·G base pair (26).

The study of mismatch repair in *E. coli* has been greatly enhanced by an in vitro repair system which monitors the conversion of a mismatch in a bacteriophage fl heteroduplex to restriction sensitivity (27). Because *S. typhimurium* cell extracts contain a nuclease activity that degrades input DNA, we were not able to demonstrate mismatch repair by this assay. Therefore, a two-step procedure for detection of A/G mismatch correction was developed in which repair was initiated with *S. typhimurium* cell extracts in the absence of Mg^{2+}. After the removal of cell extracts, the reaction was completed by adding the Klenow fragment of DNA polymerase I and T4 DNA ligase. By this in vitro assay we have shown that the *mutB* gene product of *S. typhimurium* is involved in an A/G-specific repair pathway. We have also accurately mapped this poorly characterized mutator locus and determined its mutational specificity. Our results indicate that the *S. typhimurium mutB* gene may be homologous to the *E. coli micA* (and/or *mutY*) gene.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. *S. typhimurium* and *E. coli* bacterial strains used in this work are shown in Table 1. P22 *int3HT* was obtained from Eric Eisenstadt and used in all transduction experiments. Plasmid pGD104 is a derivative of pBR322 in which codon 70 of the *bla* gene was changed from a serine (AGC) to an arginine (CGC) codon by oligonucleotide-directed mutagenesis (14). Bacteriophages fl R229, with one single *Eco*RI sequence (6), fl ALLC3, with a dC-to-dA base substitution, and fl M28 (from Paul Modrich) with a dC-to-dT base substitution at position 5621 have been described previously (25, 27). Covalently closed heteroduplexes of fl DNA containing an A/G or T/G mismatch within the *Eco*RI recognition site were prepared from replicative-form DNA of fl R229 and single-stranded DNA of fl ALLC3 or fl M28, respectively, as described previously (27).

Media. LB medium and M9 minimal medium were as described by Miller (31). Sugars were added to M9 medium at a final concentration of 0.2% (wt/vol), and amino acids were added at a final concentration of 40 μg/ml. Tetracycline was used at a final concentration of 20 μg/ml, and kanamycin and ampicillin were added to media to give a final concentration of 50 μg/ml. MacConkey indicator plates contained (per liter) 8.0 g of nutrient broth, 15.0 g of agar, 100 mg of neutral red, and 10.0 g of the appropriate sugar.

Strain construction. WS6 and WS7 were constructed by infecting TA4011 and TA4016, respectively, with a transducing lysate grown on GW1803 and selecting kanamycin-resistant (Km^r) transductants. WS14 and WS15 were isolated as tetracycline-resistant (Tc^r) transductants of GW1809 and GW45, respectively, following infection with a transducing lysate of TT184. The *endA41*::Tn*10* allele was introduced into GW2 and DS1373 by selecting Tc^r transductants resulting from infection with a RED10 transducing lysate. The resulting strains are WS38 and WS10, respectively.

pGD104 DNA, the gift of P. Foster, was introduced into the r^- m^+ strain LB5010 by using the $CaCl_2$ transformation procedure of Lederberg and Cohen (20). pGD104 carrying *S. typhimurium* modification was isolated from LB5010 and used to transform GW45 and GW1809. All pGD104 transformants were selected on LB-tetracycline agar. GW45 (pGD104) and GW1809(pGD104) were used in the reversion assays described below.

TABLE 1. Bacterial strains used in this work[a]

Strain	Genotype	Source (reference)
S. typhimurium		
GW2	*hisG46 gal-6*	G. Walker (44)
GW45	*hisG46*	G. Walker (44)
GW1702	*hisG46 mutS121*::Tn*10*	G. Walker (44)
GW1803	*hisG46 gal-6 mutB131*::Tn*5*	G. Walker (44)
GW1809	*hisG46 mutB131*::Tn*5*	G. Walker (44)
GW1816	*hisG46 mutS121*::Tn*10* *mutB131*::Tn*5*	G. Walker (44)
TA4011	*hisG1775*	B. Ames (21)
TA4016	*hisC9070*	B. Ames (21)
LB5010	*ilv leu met trp* r^-m^+ *galE*	P. Foster
DS1373	*metK36*	SGSC[b]
RED10	*endA41*::Tn*10*	SGSC
TT184	*proBA662*::Tn*10*	SGSC
WS6	*hisG1775 mutB131*::Tn*5*	This laboratory
WS7	*hisC9070 mutB131*::Tn*5*	This laboratory
WS10	*metK36 endA41*::Tn*10*	This laboratory
WS14	*hisG46 mutB131*::Tn*5* *proBA662*::Tn*10*	This laboratory
WS15	*hisG46 proBA662*::Tn*10*	This laboratory
WS38	*hisG46 gal-6 endA41*::Tn*10*	This laboratory
E. coli		
CC101	F′ *lacI lacZ* (Am-461) *proA*$^+$ *proB*$^+$ (*lac proB*) *thi strA*	J. Miller (34)
CC102	CC101 but *lacZ* (Gly-461)	J. Miller (34)
CC103	CC101 but *lacZ* (Gln-461)	J. Miller (34)
CC104	CC101 but *lacZ* (Ala-461)	J. Miller (34)
CC105	CC101 but *lacZ* (Val-461)	J. Miller (34)
CC106	CC101 but *lacZ* (Lys-461)	J. Miller (34)
M182 (PR8)	*lacZX74 galU galK* Sm^r	E. Signer
PR9	M182 *mutL218*::Tn*10*	M. Fox (38)
PR68	M182 *mutL218*::Tn*10* *micA68*::modified Tn*10*	M. Fox (38)
PR70	M182 *micA68*::modified Tn*10*	M. Fox (38)

[a] The nomenclature is that of Sanderson and Roth (42) and Bachmann (3).
[b] SGSC, *Salmonella* Genetic Stock Center.

Each of the F′ episomes contained in strains CC101 to CC106 was introduced into WS14 and WS15 by the following conjugation procedure. Samples (0.1 ml) of overnight cultures of the appropriate donor and recipient strains were spread together on an LB plate, which was then incubated for 12 to 16 h at 37°C. Cells were harvested in 3.0 ml of 0.85% NaCl, and 0.1 ml of a 1:40 dilution of this cell suspension was plated on M9 glucose agar supplemented with histidine and tetracycline to select Pro^+ Tc^r exconjugants. These strains were used in the Lac^+ reversion assays.

Transductional mapping. Exponentially growing recipient cells were infected with the appropriate P22 *int3HT* transducing lysate at a multiplicity of infection of 0.005 to 0.01, incubated at 42°C (to decrease Tn*5* transposition events) for 30 min, and plated on LB-kanamycin or LB-tetracycline plates. For crosses in which *endA*$^+$ was the unselected marker, the Km^r transductants were toothpicked onto LB medium containing kanamycin and tetracycline. For crosses in which *metK*$^+$ was an unselected marker, the Km^r or Tc^r transductants were toothpicked onto M9 glucose containing kanamycin or tetracycline, respectively, plus 90 μg of ethionine per ml and 60 μg of norleucine per ml. *metK*$^+$ cells are sensitive to ethionine and norleucine at 42°C, whereas *metK* cells are resistant. In the transductional cross of GW1803 × WS38, all Km^r transductants were checked for the mutator

phenotype by patching cells onto MacConkey galactose-kanamycin plates and monitoring Gal^+ papilla formation.

Reversion assays. Independent cultures of each strain were grown to stationary phase in LB medium starting from an initial inoculum of less than 200 cells. For derivatives of WS14 and WS15 containing F'*lacZ* episomes, 1.0 ml of cells from each culture was pelleted and suspended in 1.0 ml of 0.85% NaCl before 0.1 ml of cells was plated onto M9 lactose agar containing histidine to select Lac^+ revertants. For these strains, exhibiting a very low Lac^+ reversion frequency, cells were concentrated threefold in 0.85% NaCl before 0.1 ml was plated onto M9 lactose agar. The cell titer of each culture was determined by plating a 10^{-6} dilution onto LB agar. Colonies from the titer plates were replica plated onto M9 glucose agar supplemented with histidine to ensure that the cells had retained the Pro^+ phenotype conferred by the episome. The frequency of His^+ revertants for strains bearing the *hisG1775* or *hisC9070* allele was measured as described previously (44). The frequency of ampicillin-resistant (Ap^r) revertants for GW45(pGD104) and GW1809 (pGD104) was determined by plating 0.1 ml of each culture onto LB-ampicillin plates and measuring the cell titer as described above.

Labeling of DNA substrates. A 120-base-pair (bp) *Hae*II-*Taq*I fragment (coordinates 5572 to 5691) spanning the *Eco*RI site of bacteriophage fl derivatives was purified and 3' end labeled at the *Taq*I terminus by using the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP (29). A 162-bp *Hin*PI-*Sau*96I fragment (coordinates 5569 to 5730) was used for 3' end labeling at the *Hin*PI terminus by the same procedure. A 265-bp *Hgi*AI-*Sau*96I DNA fragment (coodinates 5465 to 5730) was 5' end labeled at the *Hgi*AI terminus by using [γ-^{32}P]ATP (3,000 Ci/mmol) and polynucleotide kinase (29). DNA fragments labeled at only one end were obtained from such preparations by secondary restriction cleavage and isolation of the desired products by electrophoresis on nondenaturing polyacrylamide gels.

A synthetic 40-mer oligonucleotide, 5'-AATTGGGCT CCTCGAGGAATT<u>N</u>GCCTTCTGCAGGCATGCC-3', was annealed with its complementary oligonucleotide 5'-CCGG GCATGCCTGCAGAAGGC<u>G</u>AATTCCTCGAGGAGCCC-3', where N represents A, T, or C. The resulting duplex oligonucleotide containing an A/G, T/G, or C·G at position 21 was labeled at the 3' end by using the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP.

Extract and protein preparation. Cell extracts of *S. typhimurium* and *E. coli* were prepared as previously described for *E. coli* (27), except that the supernatant after ultracentrifugation was precipitated with 0.277 g of ammonium sulfate per ml. *E. coli* fraction V containing both A/G-binding and -nicking activities was purified by column chromatography over phophocellulose and hydroxylapatite as described by Lu and Chang (26) followed by heparin-agarose (Lu et al., in preparation).

Mismatch repair assay. The mismatch repair assay scores the conversion of fl heteroduplexes, containing a 1-bp mismatch within the *Eco*RI site, to *Eco*RI endonuclease sensitivity (27). Reactions (final volume, 20 µl) contained 0.02 M Tris hydrochloride (pH 7.6), 50 µg of bovine serum albumin per ml, 1 mM glutathione, 0.2 mM spermidine, 1.5 mM ATP, 0.5 mM NAD, 5 µg of DNA per ml, and cell extract (8 to 27 µg of protein). After the mixture had been incubated for 30 min at 37°C, 80 µl of 10 mM Tris hydrochloride (pH 7.6)–1 mM EDTA was added, and samples were extracted twice with phenol and twice with diethyl ether. DNA, collected by ethanol precipitation, was subjected to a DNA synthesis and ligation reaction, which was performed in 15 µl of 7 mM Tris hydrochloride (pH 7.6)–7 mM $MgCl_2$–50 mM NaCl–2 mM dithiothreitol–100 µM (each) deoxynucleoside triphosphates–70 µm ATP–1 U of *E. coli* DNA polymerase I (Klenow fragment)–1 U of T4 DNA ligase. Incubation was carried out for 15 min at 0°C and then at 20°C for 120 min. After heating at 65°C for 5 min and addition of Tris hydrochloride (pH 7.6) to a final concentration of 100 mM, DNA was hydrolyzed with *Eco*RI and *Bam*HI and analyzed by electrophoresis on a 1% agarose gel.

Gel-binding analysis. Protein-DNA complexes were analyzed on low-ionic-strength polyacrylamide gels as described previously (26). Protein samples were incubated with 0.9 fmol (2,000 to 6,000 cpm) of end-labeled 120-bp *Hae*II-*Taq*I or 40-bp oligonucleotide duplex DNA fragment in the presence of 20 ng of poly(dI-dC) (Pharmacia, Inc.) in a final volume of 20 µl. Binding reactions and gel electrophoresis were carried out as described by Lu and Chang (26). The gels were transferred to Whatman 3MM paper and dried prior to autoradiography.

Endonuclease nicking assay. Protein samples were incubated with end-labeled DNA fragments similarly to the binding reaction described above, except that no poly(dI-dC) was added in the reaction mixtures. In addition to the 3'-end-labeled 120-bp *Hae*II-*Taq*I DNA fragment, a 5'-end-labeled 265-bp *Hgi*AI-*Sau*96I fragment and a 3'-end-labeled 162-bp *Hin*PI-*Sau*96I fragment were also used in the nicking assay. After incubation, the samples were lyophilized and redissolved in 3 µl of 90% (vol/vol) formamide–10 mM EDTA–0.1% (wt/vol) xylene cyanol–0.1% (wt/vol) bromophenol blue. DNA was denatured at 90°C for 3 min and applied to a standard 8% polyacrylamide–8.3 M urea sequencing gel for electrophoresis (30).

RESULTS

***mutB* maps to the 64-minute region.** Prior genetic analysis of the *mutB* gene indicated that it is located in the 62- to 72-min region of the *S. typhimurium* chromosome (44). The mapping data summarized in Table 2 show that the *mutB131*::Tn*5* allele is cotransducible with two genes (*metK* and *endA*) located in the 63- to 64-min interval. Based on two-factor crosses, the *mutB* gene is located closer to the *endA* gene (32% cotransduction frequency) than to the *metK* gene (5% cotransduction frequency) and the likely gene order is *metK-endA-mutB*. The latter point was confirmed by a three-factor cross in which Km^r transductants (*mutB131*::Tn*5*) were selected in a *metK36 endA41*::Tn*10* strain and screened for *metK* and *endA* alleles (Table 2). The presence of only one Km^r Tc^r $metK^+$ transductant among the 555 colonies screened is consistent only with the gene order *metK-endA-mutB*. Using a modified form of the Wu equation (42) which corrects for the effect of insertion mutations on cotransduction frequency, the *mutB* gene is located at 63.7 min on the *S. typhimurium* genetic map. This location is strikingly similar to that determined for the *E. coli micA* (and/or *mutY*) gene (34, 38).

***mutB131*::Tn*5* specifically increases C·G-to-A·T transversions.** Cupples and Miller (10) have recently described a set of six *lacZ* mutations at codon 461 which can be used to analyze mutational spectra. For each allele, Lac^+ revertants result from a unique transition or transversion event which restores the wild-type glutamic acid codon. These *lacZ* alleles were introduced into isogenic $mutB^+$ and *mutB131*::Tn*5* strains on F' episomes and the Lac^+ reversion fre-

TABLE 2. Genetic linkage of *mutB* with *endA* and *metK* as determined by P22 transduction experiments

Donor (relevant genotype)	Recipient (relevant genotype)	Transductant class[a]	No. of transductants	Contransduction frequency (%)
GW1803 (*mutB131*::Tn*5*)	WS38 (*endA41*::Tn*10*)	Km^r Tc^r	131	32
		Km^r Tc^s	61	
GW1803 (*mutB131*::Tn*5*)	DS1373 (*metK36*)	Km^r *metK36*	380	5
		Km^r *metK*$^+$	20	
RED10 (*endA41*::Tn*10*)	DS1373 (*metK36*)	Tc^r *metK36*	147	58
		Tc^r *metK*$^+$	202	
GW1803 (*mutB131*::Tn*5*)	WS10 (*endA41*::Tn*10 metK36*)	Km^r Tc^r *metK36*	399	
		Km^r Tc^s *metK36*	114	
		Km^r Tc^s *metK*$^+$	41	
		Km^r Tc^r *metK*$^+$	1	

[a] The first phenotype listed was the selected marker in the transduction experiment. Km^r is 100% linked to the *mutB* phenotype. Tc^r transductants carry the *endA41*::Tn*10* allele, whereas Tc^s transductants are *endA*$^+$.

quency of each strain was determined. These data are presented in Table 3 and show that the *mutB131*::Tn*5* allele increased the frequency of Lac$^+$ revertants arising from C·G-to-A·T transversions greater than 40-fold. There was no detectable effect of *mutB131*::Tn*5* on the frequency of Lac$^+$ revertants which result from the other five classes of base substitution mutations.

To ensure that the observed increase in C·G-to-A·T transversions in the *mutB131* background was not due to some peculiarity of the *lacZ* (Ala-461) allele, the effect of this mutator on C·G-to-A·T events at another locus was examined. pGD104 carries a mutant allele of the *bla* gene such that the only base substitution mutation that results in a fully Ap^r phenotype is a specific C·G-to-A·T transversion at codon 70 (14). The *mutB131*::Tn*5* allele increased the frequency of this C·G-to-A·T transversion in the *bla* gene more than 40-fold (Table 3). Lastly, the *mutB131*::Tn*5* allele did not affect the reversion frequency of the *hisG1775* or *hisC9070* mutations which have been reported to revert by T·A to C·G and C·G to G·C base changes, respectively (21). This preliminary analysis of the specificity of the *mutB* mutator phenotype indicates that only the frequency of C·G-to-A·T transversions is enhanced. These data are very similar to the results reported for *E. coli mutY* strains (34) and prompted the biochemical experiments reported below.

TABLE 3. Specificity of the *mutB* mutator phenotype

Genetic background	Revertible allele	Base change required	No. of revertants/10^8 cells[a]
mutB$^+$	*lacZ* (Am-461)	T·A→G·C	<0.1[b] (2/10)
mutB131::Tn*5*	*lacZ* (Am-461)	T·A→G·C	0.1[b] (6/10)
mutB$^+$	*lacZ* (Gly-461)	C·G→T·A	0.8 (8/10)
mutB131::Tn*5*	*lacZ* (Gly-461)	C·G→T·A	0.5 (6/10)
mutB$^+$	*lacZ* (Gln-461)	C·G→G·C	<0.1[b] (2/10)
mutB131::Tn*5*	*lacZ* (Gln-461)	C·G→G·C	0.5[b] (1/10)
mutB$^+$	*lacZ* (Ala-461)	C·G→A·T	0.4 (4/10)
mutB131::Tn*5*	*lacZ* (Ala-461)	C·G→A·T	19.0 (10/10)
mutB$^+$	*lacZ* (Val-461)	T·A→A·T	0.3 (4/10)
mutB131::Tn*5*	*lacZ* (Val-461)	T·A→A·T	0.4 (4/10)
mutB$^+$	*lacZ* (Lys-461)	T·A→C·G	0.1 (2/10)
mutB131::Tn*5*	*lacZ* (Lys-461)	T·A→C·G	0.1 (1/10)
mutB$^+$	*bla* (Arg-70)	C·G→A·T	0.5 (4/8)
mutB131::Tn*5*	*bla* (Arg-70)	C·G→A·T	24.0 (10/10)
mutB$^+$	*hisG1775*	T·A→C·G	<0.1 (1/5)
mutB131::Tn*5*	*hisG1775*	T·A→C·G	0 (0/5)
mutB$^+$	*hisC9070*	C·G→G·C	0.1 (2/5)
mutB131::Tn*5*	*hisG9070*	C·G→G·C	0.1 (2/5)

[a] The numbers in parentheses represent the number of independent cultures in which one or more revertants was observed divided by the number of independent cultures analyzed.

[b] Overnight cultures from these strains were concentrated threefold in 0.85% NaCl before 0.1 ml was spread on M9-lactose plates to select Lac$^+$ revertants.

S. typhimurium has a mutB-dependent A/G mismatch repair system. The in vitro assay for mismatch repair developed for *E. coli* (27) is based on repair of heteroduplex DNA of fl R229, which contains a 1-bp mismatch within the single *Eco*RI site of the molecule. The hybrid *Eco*RI site can be converted to an *Eco*RI-sensitive form when the strand bearing the mutant *Eco*RI sequence is repaired with cell extract. Using the same approach, we were not able to demonstrate mismatch repair in vitro with *S. typhimurium* cell extracts owing to the degradation of input DNA by nuclease in the crude extracts. To avoid this problem, we developed a two-step repair assay. Repair was initiated with cell extracts in the absence of Mg^{2+}, and the reaction was completed by addition of the Klenow fragment of DNA polymerase I and T4 DNA ligase after the removal of cell extracts. After these two steps, fl DNA was digested with *Eco*RI and *Bam*HI and analyzed by agarose gel electrophoresis. Heteroduplex fl DNAs with an A/G or T/G mismatch at position 5621 were used as substrates to detect A/G-to-C·G or T/G-to-C·G repair, respectively. By this in vitro assay we showed that both *E. coli* and *S. typhimurium* extracts are able to repair an A/G mismatch to a C·G base pair and that the repair is not dependent on the *E. coli mutL* or *S. typhimurium mutS* gene function (Fig. 1, lanes 1, 2, 5, and 7; Table 4). The T/G mismatch was not repaired in this two-stage in vitro assay (Fig. 1, lanes 3, 4, 9, and 11). Based on the design of the in vitro assay, the repair of A/G but not T/G mismatches does not require Mg^{2+} for the incision and excision steps and resynthesis is carried out by DNA polymerase I.

Since the *E. coli* MicA (and/or MutY) protein is involved in the *dam*- and *mutHLSU*-independent A/G-specific repair pathway (2, 38; Lu et al., in preparation), we tested the in vitro repair activity in *mutB* extracts. In the two-step repair assay, the *mutB* (GW1803) and *mutB mutS* (GW1816) mutant extracts were defective in A/G mismatch repair (Fig. 1, compare lane 5 with lane 6 and lane 7 with lane 8; Table 4). This result further supports the notion that the *mutB* gene of *S. typhimurium* is the homolog of the *E. coli micA* (and/or *mutY*) gene whose product plays a role in maintenance of replication fidelity by repair of A/G mismatches to C·G base pairs.

***mutB* extracts lack an A/G mismatch-binding protein.** To detect *S. typhimurium* proteins that specifically bind DNA fragments containing an A/G mismatch, we have used a gel

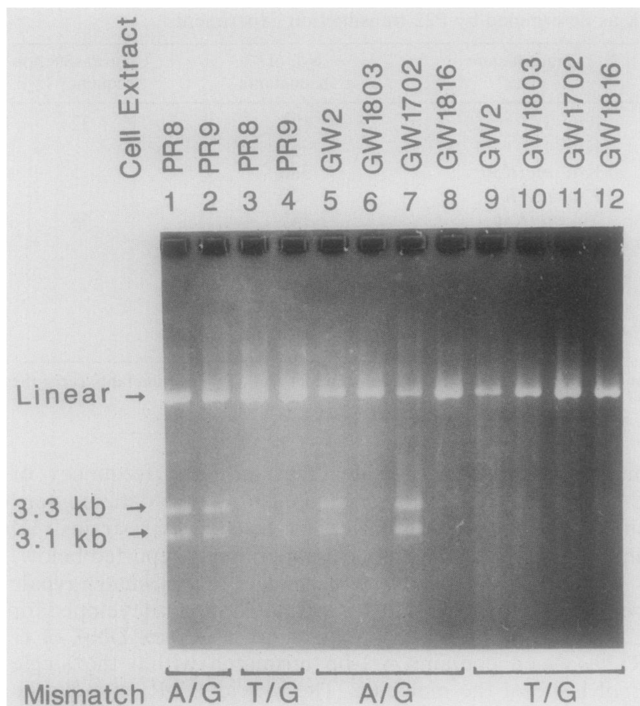

FIG. 1. In vitro mismatch repair with *S. typhimurium* cell extracts. The mismatch repair assay (27) used fl heteroduplexes containing an A/G (lanes 1, 2 and 5 to 8) or T/G (lanes 3, 4, and 9 to 12) mismatch at position 5621 within the *Eco*RI site. Hemimethylated DNA was methylated at GATC sequences on the complementary strand that contained the wild-type *Eco*RI sequence. DNA substrates were incubated with cell extracts and purified (see Materials and Methods). After DNA systhesis and ligation, DNA was cleaved by *Eco*RI and *Bam*HI and fractionated on 1% agarose gels. Digestion of fl R229 duplex DNA with *Eco*RI and *Bam*HI yields 3.1- and 3.3-kbp fragments. Repair at the mismatched site renders the site sensitive to *Eco*RI cleavage. Cell extracts used were isolated from PR8 (lanes 1 and 3), PR9 (*mutL*) (lanes 2 and 4), GW2 (lanes 5 and 9), GW1803 (*mutB*) (lanes 6 and 10), GW1702 (*mutS*) (lanes 7 and 11), and GW1816 (*mutB mutS*) (lanes 8 and 12).

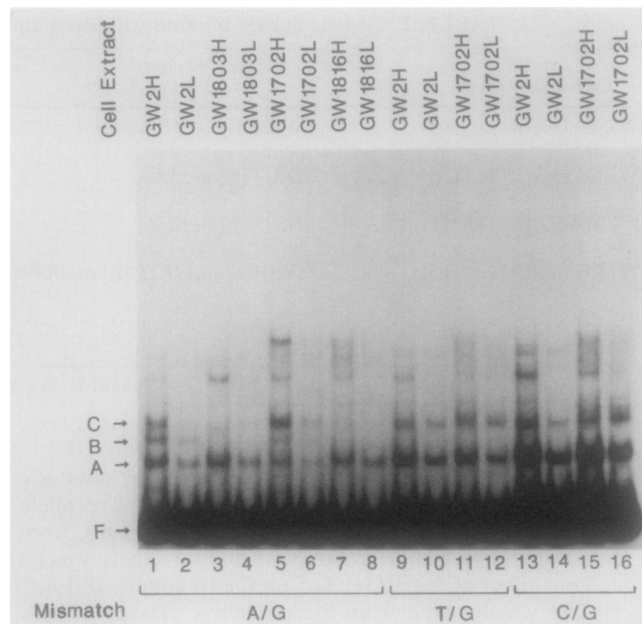

FIG. 2. Detection of specific DNA-binding proteins for A/G mismatch of 120-bp fragment is *S. typhimurium* cell extracts. The labeled 120-bp *Hae*II-*Taq*I fragment with an A/G (lanes 1 to 8), T/G (lanes 9 to 12), or no (C·G; lanes 13 to 16) mismatch at position 5621 was incubated with protein extracts. DNA after incubation was fractionated by electrophoresis through a low-ionic-strength 4% polyacrylamide gel. Several distinct bands (A, B, and C) of lower mobility were observed. F represents the position of free DNA. Cell extracts used were GW2 (0.75 μg; lanes 1, 9, and 13; 0.38 μg, lanes 2, 10, and 14), GW1803 (*mutB*) (1 μg, lane 3; 0.5 μg, lane 4), GW1702 (*mutS*) (0.8 μg, lanes 5, 11, and 15; 0.4 μg, lanes 6, 12, and 16), and GW1816 (*mutB mutS*) (0.75 μg, lane 7; 0.38 μg, lane 8). H and L represent the higher and lower concentrations of the cell extracts.

TABLE 4. Requirement for the *mutB* gene product in specific A/G-to-C·G mismatch repair[a]

Heteroduplex	Cell extract	*Eco*RI site repaired (%)[b]
A. G A A T T [T] C T T A A [G]	PR8 (wild type)	0
	PR9 (*mutL*)	0
	GW2 (wild type)	0
	GW1803 (*mutB*)	0
	GW1702 (*mutS*)	0
	GW1816 (*mutS mutB*)	0
B. G A A T T [A] C T T A A [G]	PR8 (wild type)	45
	PR9 (*mutL*)	47
	GW2 (wild type)	33
	GW1803 (*mutB*)	0
	GW1702 (*mutS*)	71
	GW1816 (*mutS mutB*)	0

5616 5621

[a] fl DNA GAATGC . . . . viral
*Eco*RI site CTTAAG . . . . complementary, shows the *Eco*RI site (at positions 5616 to 5621) of the replicative form of fl R229.
[b] Percentages of input DNA being repaired.

electrophoresis DNA-binding assay (8, 15) to detect an A/G mismatch binding protein in *E. coli* (26). Binding to DNA fragments containing an A/G mismatch was compared with binding to DNA substrates containing a T/G mismatch or no mismatch. Labeled 120-bp *Hae*II-*Taq*I fragments isolated from fl heteroduplexes containing an A/G, T/G, or C·G at position 5621 were incubated with protein extracts of *S. typhimurium* and fractionated by gel electrophoresis. In these assays, several distinct DNA-protein complexes (A, B, C, etc.) of low mobility were observed (Fig. 2, lanes 1, 2, 5, 6, and 9 to 16). The protein comprising complex B had higher affinity for A/G mismatch-containing DNA than for T/G- or C·G-containing DNA (Fig. 2, compare lanes 1 and 2 with lanes 9, 10, 13, and 14).

In addition, labeled 40-bp oligonucleotide duplexes (see Materials and Methods) containing an A/G, T/G, or C·G at position 21 were used in the gel retardation DNA-binding assay as above. Similarly, several DNA-protein complexes were formed, as observed by their lower mobility (Fig. 3). In this case, two complexes (B′ and C′) were specific to the A/G mismatch-containing DNA (Fig. 3, lanes 2 to 4). The complex C′ had the same mobility as the complex formed with fraction V of *E. coli*, which possesses a partially purified A/G-specific binding activity (see Materials and Methods) (Lu et al., in preparation) (Fig. 3, lane 1). These observations suggest that *S. typhimurium* cell extracts contain one or more proteins which specifically bind A/G mismatch-containing DNA and that one of the binding proteins is comparable in size to the one found in *E. coli*.

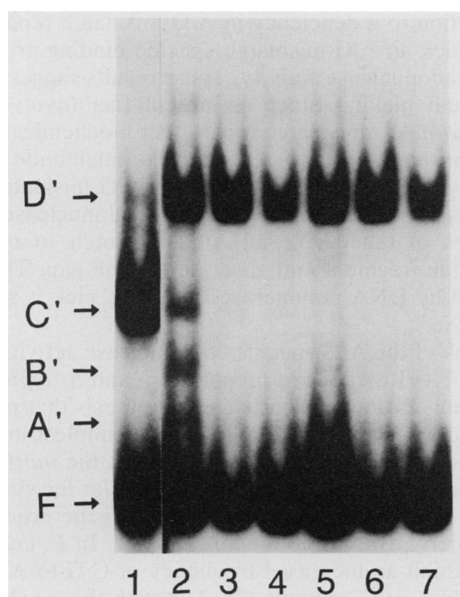

FIG. 3. Detection of specific DNA-binding proteins for A/G mismatch of the 40-bp fragment in *S. typhimurium* cell extracts. The binding reaction was carried out as for Fig. 2, except that a labeled 40-bp oligonucleotide duplex with an A/G (lanes 1, 2, and 5), T/G (lanes 3 and 6) or no (lanes 4 and 7) mismatch was incubated with protein extracts from GW1702 (*mutS*) (0.8 μg, lanes 2 to 4), and GW1816 (*mutB mutS*) (0.75 μg, lanes 5 to 7). The oligonucleotide with an A/G mismatch was also incubated with fraction V from *E. coli* (lane 1 [Lu et al., in preparation]). Several distinct bands (A', B', C', and D') of lower mobility were observed. F represents the position of free DNA.

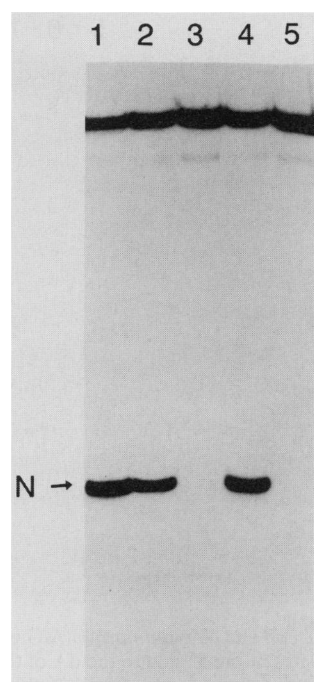

FIG. 4. Deficiency of A/G endonuclease activity in *mutB* mutant extracts. A 120-bp *Hae*II-*Taq*I fragment with an A/G mismatch at position 5621 and labeled at the 3' end of the *Taq*I terminus was incubated with fraction V from *E. coli* (lane 1 [Lu et al., in preparation]) and different cell extracts [lane 2, GW2; lane 3, GW1803 (*mutB*); lane 4, BW1702 (*mutS*); and lane 5, GW1816 (*mutB mutS*)]. Reaction products after denaturation were analyzed on an 8% polyacrylamide DNA sequencing gel. N indicates the cleavage product of A/G endonuclease. The *mutB* mutant extracts (GW1803 and GW1816) have no A/G endonuclease activity.

To test whether the A/G mismatch-specific binding activity of *S. typhimurium* is dependent on the *mutB* gene product, we assayed binding activity with *mutB* mutant extracts. DNA-protein complex B or complexes B' and C' were absent when *mutB* mutant extracts were incubated with the 120-bp *Hae*II-*Taq*I f1 DNA fragment (Fig. 2, lanes 3, 4, 7, and 8) or the 40-bp oligonucleotide duplex (Fig. 3, lane 5) containing an A/G mismatch, respectively. Thus, the A/G mismatch-specific binding complexes were not observed in GW1803 (*mutB*) or GW1816 (*mutS mutB*) mutant extracts.

***mutB* extracts are deficient in an A/G mismatch-specific endonuclease.** An A/G mismatch-specific endonuclease was also found in *S. typhimurium* crude cell extracts. The 120-bp *Hae*II-*Taq*I fragment labeled at the 3' end of the *Taq*I terminus was incubated with protein extracts and analyzed on a denaturing sequencing gel (30). An endonuclease which cuts at a specific site will generate a discrete fragment which migrates faster than the uncleaved substrate. The endonuclease was specific for A/G-containing fragments (Fig. 4, lanes 2 and 4) and had no cleavage activity on T/G- or C·G-containing fragments (data not shown).

Like the A/G endonuclease found in *E. coli* (Fig. 4, lane 1) (26), this *S. typhimurium* endonuclease did not require Mg^{2+} and made incisions at the same sites on an A/G-containing DNA fragment. We have used the 120-bp *Hae*II-*Taq*I fragment (Fig. 4) or 265-bp *Hgi*AI-*Sau*96I fragment (Fig. 5) to determine the cleavage sites of the A/G mismatch-specific endonuclease. The denatured cleavage products were separated on a sequencing gel in parallel with the cleavage product of fraction V of *E. coli*. The cleavage products of the *S. typhimurium* endonuclease ran at the same position as those of the *E. coli* enzyme when the 120-bp *Hae*II-*Taq*I labeled at the 3' end of the *Taq*I terminus was used as the substrate (Fig. 4). Both *S. typhimurium* and *E. coli* A/G endonucleases also had the same incision sites on the 265-bp *Hgi*AI-*Sau*96I DNA fragment labeled at the 5' end of the *Hgi*AI terminus (Fig. 5). There was no detectable incision site on the other DNA strand (with dG at position 5621) when the 162-bp *Hin*PI-*Sau*96I fragment labeled at the 3' end of the *Hin*PI terminus was used (data not shown). Therefore, the A/G endonuclease of *S. typhimurium* presumably made two incisions on the DNA strand which contains the dA of the A/G mismatch and had no detectable incision site on the other strand. The endonuclease cleaved the first phosphodiester bond 3' to and the second phosphodiester bond 5' to the dA of the A/G mismatch (26).

Extracts prepared from *E. coli micA* mutants do not contain an A/G mismatch-specific endonuclease activity (Fig. 5, lanes 2 and 4) (Lu et al., in preparation); therefore, nicking activity was assayed with *mutB* mutant extracts. The specific nicking activity was not observed in GW1803 (*mutB*) or GW1816 (*mutS mutB*) extracts with either the 120-bp *Hae*II-*Taq*I (Fig. 4, lanes 3 and 5) or the 265-bp *Hgi*AI-*Sau*96I (Fig. 5, lanes 6 and 8) fragment as substrate.

DISCUSSION

The in vitro assay for mismatch repair developed in *E. coli* (27) has been used to demonstrate the existence of two distinct repair pathways in this organism. The methyl-directed mismatch repair system acts on a variety of base

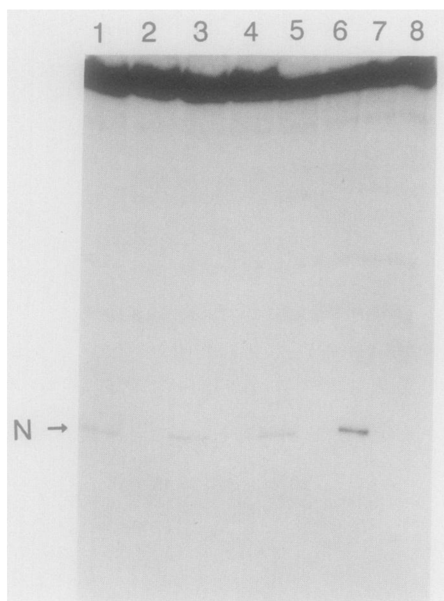

FIG. 5. Both *E. coli* and *S. typhimurium* A/G endonucleases had the same incision sites at the 5′ side to the dA of the A/G mismatch. Experiments were performed as described in the legend to Fig. 4, except that the 265-bp *Hgi*AI-*Sau*96I fragment containing an A/G mismatch at position 5621 was labeled at the 5′ end of the *Hgi*AI terminus. Cell extracts used were PR8 (lane 1), PR70 (*micA*; lane 2), PR9 (*mutL*; lane 3), PR68 (*micA mutL*; lane 4), GW2 (lane 5), GW1803 (*mutB*; lane 6), GW1702 (*mutS*; lane 7), and GW1816 (*mutB mutS*; lane 8).

mismatches and is dependent on *dam* methylation, ATP, and the gene products of *mutHLS* and *uvrD* (27). The other pathway is independent of all of the above three factors (25, 38, 45), requires the *micA* (and/or *mutY*) gene product (2, 38; Lu et al., in preparation), and acts specifically on A/G mismatches. We now describe an adaptation of the in vitro assay which enables the detection of an A/G mismatch repair activity in cell extracts of *S. typhimurium*. The major modification is the initiation of the repair process in the absence of Mg^{2+} which overcomes the problem of nonspecific nucleases in *S. typhimurium* extracts. The reaction is completed by the addition of the Klenow fragment of DNA polymerase I and T4 DNA ligase after the removal of the cell extracts.

Several lines of evidence suggest that the repair detected in *S. typhimurium* by this in vitro assay is equivalent to the A/G mismatch-specific repair reported in *E. coli* (25, 45). The repair in *S. typhimurium* extracts is independent of ATP, *dam* methylation (data not shown), and the gene product of *mutS*. Both *E. coli* and *S. typhimurium* extracts contain an endonuclease activity associated with A/G mismatch repair which cleaves the first phosphodiester bond 3′ to the dA of an A/G mismatch and also the second phosphodiester bond 5′ to the same dA. In both organisms, this activity does not require Mg^{2+} and is specific for A/G mismatches. Lastly, the *S. typhimurium* A/G-specific repair system is dependent on the product of the *mutB* gene, which is very likely to be a homolog of the *E. coli micA* (and/or *mutY*) gene. The latter conclusion is based on our genetic evidence that the *mutB131* allele maps at 63.7 min on the *S. typhimurium* chromosome and specifically increases C·G-to-A·T transversions. Both of these characteristics are identical to those reported for *mutY* strains (34).

In addition to a deficiency in A/G mismatch repair, *mutB* extracts lack an A/G mismatch-specific binding protein and the A/G endonuclease activity. These results suggest that the binding and nicking functions are in fact involved in *S. typhimurium* A/G mismatch repair. Our biochemical data for *S. typhimurium* are consistent with the nucleotide excision repair model previously described for A/G mismatch repair in *E. coli* (27). In this model, the A/G endonuclease cuts on both sides of the dA of an A/G mismatch to remove a dinucleotide fragment and generate a small gap. The gap is then filled by DNA polymerase I, and the nick is sealed by DNA ligase.

The lack of the A/G-specific endonuclease activity in both *E. coli micA* (Lu et al., in preparation) and *S. typhimurium mutB* strains further supports our hypothesis that *mutB* and *micA* (and/or *mutY*) are homologs. The simplest interpretation of the results presented above is that the *mutB* protein participates directly in the recognition and/or incision events at A/G mismatches. However, the *mutB* gene product may not be solely responsible for this activity. In *E. coli*, *mutM* strains exhibit an increased frequency of C·G-to-A·T transversions like *mutY* mutants (7). Although the *mutM* mutant is able to repair A/G mismatches to C·G base pairs in an in vitro assay in the absence of ATP (A-L. Lu, unpublished result), the *mutM* gene product has not been excluded from playing a role in A/G mismatch repair. To date, no *S. typhimurium* homolog of *mutM* has been identified. An intensive screen for new *S. typhimurium* mutators may well turn up homologs of the *E. coli mutM* and *mutT* (see below) genes.

The involvement of the *mutB* gene product in the A/G mismatch repair pathway suggests that this repair process is responsible for the reduction of C·G-to-A·T transversions. Failure to repair A/G mismatches resulting from the misincorporation of dA opposite dG residues during DNA replication neatly explains the mutator phenotype of *mutB* strains. However, a complete model must also explain how the *mutB*-dependent repair pathway is prevented from acting on A/G mismatches resulting from the misincorporation of dG opposite dA. If allowed to proceed, the latter process would transform replication errors into permanent T·A-to-G·C mutations. Lu and Chang (25) and Au et al. (2) have presented a plausible explanation for the analogous problem in *E. coli*. This model proposes that the *mutT* gene product prevents A/G mismatches with the dG in the newly synthesized strand so that the *micA* (and/or *mutY*)-dependent repair process rarely sees them. This model is attractive because it explains the mutational spectrum exhibited by *mutT* strains (specific enhancement of T·A-to-G·C transversions), a variety of observations suggesting that the *mutT* gene product acts during DNA replication (1, 5, 43; V. Desiraju and A-L. Lu, unpublished results), and the apparent lack of strand discrimination by the *micA* (and/or *mutY*)-dependent repair pathway. Consistent with this model, a *micA mutT* double mutant has a twofold-lower mutation frequency than the *mutT* single mutant (Desiraju and Lu, unpublished results). Given the absence of any *mutT* allele of *S. typhimurium*, it is currently premature to speculate whether the model developed for *E. coli* will be directly applicable to *S. typhimurium*.

In addition to its role in the avoidance of C·G-to-A·T transversions, the *mutB*-dependent repair of A/G mismatches to C·G base pairs may be involved in gene conversion in regions of heteroduplex DNA formed during genetic recombination. As a result, this repair process could also be

partially responsible for many earlier findings of high negative interference (35, 47) or map expansion (11, 16).

The *mutB*-dependent repair pathway appears to be another example of the functional equivalence of *S. typhimurium* and *E. coli* mismatch repair proteins. Both organisms also possess the *dam*-dependent pathway which requires the *mutHLS* and *uvrD* gene functions (9, 32, 36, 39). Pang et al. (36) have shown that the *S. typhimurium mutHLS* and *uvrD* genes complement defects in the corresponding *E. coli* mutants despite the likelihood that there are specific protein-protein interactions among these gene products. It will be interesting to determine whether the *micA* (and/or *mutY*) gene is capable of complementing a defect in the *S. typhimurium mutB* gene and vice versa. Preliminary results indicate that a 3.5-kbp cloned *E. coli micA* gene is able to complement the mutator phenotype of *mutB* strains (J.-J. Tsai and A-L. Lu, unpublished results). The reciprocal experiment must await the cloning of the *mutB* gene, which is in progress.

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