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Repair of Heteroduplex DNA Molecules with Multibase Loops in *Escherichia coli*

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The fate of heteroduplex molecules containing 5-, 7-, 9-, 192-, 410-, and 514-base loops after transformation of wild-type and various mutant strains of *Escherichia coli* has been examined. No evidence for repair was obtained for the wild type or for strains with mutations in the following genes: *mutS*, *recA*, *recBC sbcBC*, *recD*, *recF*, *recJ*, *recN*, *recO*, *recR*, *recBC sbcBC recF uvrA*, *recG ruvC*, *ruvB*, *lexA3*, *lexA51*, *uvrA*, *nfo xth nth*, *polA*(Ts), or *pcnB*. These results rule out the involvement of the SOS system and most known recombination and repair pathways. Repair of heteroduplex molecules containing 410- and 514-base loops was observed when a 1-base deletion-insertion mismatch was present nearby. The repair of both the mismatch and the loops was directed by the state of *dam* methylation of the DNA chains and was dependent on the product of the *mutS* gene. A high efficiency of repair (95%) was found even when the mismatch and the loops were 1,448 nucleotides apart. We conclude that multibase loops in DNA can be removed only as a consequence of corepair by *dam*-directed mismatch repair.

We define heteroduplex molecules or heteroduplexes as DNA molecules which have mismatched or unpaired bases. These heteroduplexes can be formed in vivo in several ways, e.g., by replication errors, during genetic recombination between similar but not identical DNA strands, or by spontaneous or chemical modification of bases. Heteroduplexes can replicate to generate progeny molecules with either one or the other parental genotype, or they can be substrates for specific repair systems. If repair occurs preferentially on one of the DNA chains before replication, more of the resulting products would have one of the parental genotypes.

An additional possible fate of a heteroduplex molecule was described by Meselson and coworkers (46, 47). When lambda phage heteroduplexes with a base mismatch were transfected into bacterial cells, mixed plaques with both parental genotypes were recovered at a frequency much lower (about 30%) than that expected (i.e., 100%). There was a corresponding increase in plaques with either of the pure parental genotypes. This result can be explained if only one of the two strands of the heteroduplex had been replicated preferentially in the infected cell and if the strand was selected at random. This phenomenon was termed strand loss. The molecular basis for strand loss is unknown.

One of the systems that acts on base mismatches in heteroduplex DNA is *dam*-directed mismatch repair in which the direction of strand correction is determined by the degree of N^6-adenine methylation at GATC sequences of the DNA chains (37). In contrast to strand loss, experiments with phage lambda base-mismatch heteroduplexes indicated that correction not only was dependent on strand methylation but also varied widely depending on the mismatch used (31). In addition, repair was confined to a small region of the chromosome not more than a few kilobases in length (47) and was absent in fully methylated heteroduplexes and *uvrD* and *mutL* strains of *Escherichia coli*. Similar results were obtained with phage M13 heteroduplexes which also showed a hierarchy of correction efficiency depending on the mismatch (21).

The *dam*-directed repair system of *E. coli* also corrects with high efficiency heteroduplex molecules with 1, 2, or 3 unpaired bases both in vitro (22) and in vivo (35). The frequency of correction in vivo is reduced in *mutH*, *mutL*, and *mutS* bacteria, and the direction of repair is dependent on strand methylation (35). Heteroduplex molecules with 4 unpaired bases are marginally repaired, and those with five unpaired bases are resistant to repair (35).

dam-directed mismatch repair is also active in correction of heteroduplex regions formed during recombination, since *dam*, *mutH*, *mutL*, and *mutS* strains show a hyper-recombination phenotype (12, 30). Interspecies crosses between *E. coli* and *Salmonella typhimurium* indicate that the *dam*-directed system suppresses formation of heterospecific recombinants (39).

In previous studies, we have shown that both A-C and G-T were the most frequent mismatches occurring in vivo in a target gene (*mnt*) in *dam*, *mutH*, and *mutL* strains (5, 40, 48). The last two mutant strains are defective in *dam*-directed mismatch repair. In contrast, insertion element insertion was the most frequent mutational event in wild-type bacteria, while purine-purine and pyrimidine-pyrimidine mismatches were most frequent in *mutD* (*dnaQ*) bacteria defective in DNA polymerase III exonucleolytic proofreading (48). In addition, from each strain examined above we recovered mutant bacteria which had suffered deletions of genetic material (48). These deletions were seemingly random with respect to lengths and sequences of the deletion junctions and were therefore different from those arising by recombination in flanking sequences (1). The frequency of these deletions reached 50% of all mutations after replication of the target gene in vitro by DNA polymerase III holoenzyme in the absence of single-strand-binding protein (Ssb) and transformation of the products into a mismatch-repair-defective *mutH* strain (4).

These deletions could have arisen by slippage of the polymerase on the Ssb-deficient template strand to produce heteroduplex DNA molecules containing multibase loops.

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Replication of such heteroduplex molecules would lead to the production of both wild-type and mutant progeny, provided that such structures are not repaired at high efficiency. To test this idea, we have constructed heteroduplex molecules containing multibase loops of various sizes and examined their susceptibilities to repair.

In addition, we have recently found that the frequency of mutation in a plasmid-borne gene appears to be related to the state of plasmid multimerization (3). We suggested that the high frequency of mutation in tandem multimeric plasmids may be caused by the formation of single-stranded multibase loops formed by homologous pairing out of register. In this model, such multibase loops should be relatively stable to processing by cellular enzymes. The present study was also undertaken to test this idea.

MATERIALS AND METHODS

Media. Cells were cultured in brain heart broth (20 g/liter; Difco, Detroit, Mich.) or L broth (27) solidified when required with 1.6% agar (Difco). MacConkey agar (Difco) was supplemented with 60 μg of ampicillin (Sigma, St. Louis, Mo.) per ml. Tetracycline (Sigma) was added to the media at 3.5 or 10 μg/ml; 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Bachem, Torrance, Calif.) at 40 μg/ml; and streptomycin at 100 μg/ml. The minimal medium used was that of Davis and Mingioli (9).

Plasmids. Plasmid pPY97 (40) and mutants deleted for 5 (35), 192, and 410 (4) bases in *mnt* as well as the single-base (thymine) deletion in the Mnt operator (5) have been described previously. The M13 phage origin of replication on pPY97 was used as the 514-base loop (50). pPY97 derivatives deleted for 7 and 9 bases were isolated by oligonucleotide-directed mutagenesis by using T7 DNA polymerase as described elsewhere (2). The oligonucleotides used were 5′-TGGCTAGAGATGATCTAACTTCCGTATGC-3′ and 5′-TGGCTAGAGATGATAACTTCCGTATGC-3′ (Operon Technologies, Inc., Alameda, Calif.). Plasmid DNA for sequencing was prepared as described elsewhere (40, 50). DNA sequencing (42) with Sequenase (U.S. Biochemicals, Inc., Cleveland, Ohio) used double-stranded plasmid DNA as template (6, 50). Primer MM1 (40) as well as the *Eco*RI site and *Hin*dIII site primers (New England Biolabs, Beverly, Mass.) was used. The nucleotides between the *Eco*RI and *Hin*dIII sites for each of the constructs, except the 410-base deletion, were sequenced and then subcloned into the *Eco*RI-*Hin*dIII backbone of pPY97 or pBR322. The sequence of the nucleotides between the *Sca*I and *Hin*dIII sites of the plasmid deleted for 410 bases was verified and subcloned into the backbone of pPY97 digested with the same enzymes.

Phages. The f1 phage R408 was a gift from M. Russell (41). Lambda GM110 (*imm*^{21} Δ*nin5*) which has an *mnt*::*lacZYA* fusion, was constructed essentially as described by Simons et al. (43). The *Eco*RI-*Bam*HI fragment from pPY97 was introduced into the polylinker of pRS415 (43) to produce plasmid pMQ246. Cells containing pMQ246 were infected with lambda RS45 (43), and phage recombinants carrying the *mnt*::*lacZYA* fusion were identified as those forming blue plaques on the indicator strain GM563 cultivated on L agar plates containing X-Gal.

Bacterial strains. All strains used were derivatives of *E. coli* K-12 AB1157, with the exception of MRi93 and CM5298 (Table 1). Strain GM563 is a Δ(*lac-pro*)$_{XIII}$ Leu$^+$ Ara$^+$ (Str^r) derivative of strain AB1157 obtained as a recombinant from mating with Hfr KL584 by the procedure previously described (29). GM4326 is GM563 lysogenized with lambda GM110. GM4331 was isolated as a spontaneous Lac$^+$ revertant of GM4326 on MacConkey agar. Introduction of plasmid pPY97 into GM4331 reverted the phenotype to Lac$^-$, indicating that the mutation is in the *mnt* gene. The mutational defect in this gene has not been identified further.

Pro$^+$ Δ*lacU169* (Str^r) derivatives of the *recD*, *recF*, *recJ*, *recN*, *recO*, and *recR* strains were obtained after mating with Hfr3000 Δ*lacU169*. Recombinants with Δ*lacU169* are Lac$^-$ on MacConkey agar containing 33 μM isopropyl-β-D-thiogalactopyranoside, while the F$^-$ parents with *lacY1* are Lac$^+$. The *recBC sbcBC* and *ruvB* strains were transduced to Δ*lacU169* by using *P1vir* transduction (29) with the linked *proC*::Tn*5* marker.

Strain GM5834 (*recBC sbcBC recF uvrA*) was constructed by mating GM5814 (*recBC sbcBC*) with Hfr GM2801 (*uvrA recF tna*::Tn*10*) and selecting for Tet^r (Str^r) recombinants and subsequent screening for sensitivity to UV irradiation.

Cells lysogenic for lambda GM110 were isolated by infection of stationary-phase cultures in 10 mM $MgSO_4$ with phage for 20 min at 37°C followed by dilution and plating on L agar with X-Gal. Blue colonies were tested for superinfection immunity with lambda GM110 and lambda *vir*. Several lysogens from each strain were assayed for β-galactosidase activity as described by Miller (32) to ensure the isolation of monolysogens.

Preparation of heteroduplex molecules. Plasmid DNA mutated in *mnt* was isolated by using Qiagen (Chatsworth, Calif.) maxi-columns according to the manufacturer's instructions. Single-stranded (wild-type) DNA was isolated as described elsewhere (14) from phage R408 lysates of strain GM2622. Covalently closed supercoiled heteroduplexes with 5, 7, 9, 192, 410, or 514 loops were constructed by the method described by Lu et al. (26). Plasmid DNA with a mutation in *mnt* was linearized with *Pvu*II, denatured with alkali, and renatured in the presence of a fivefold molar excess of single-stranded (wild-type) DNA. The nicked circular heteroduplex molecules were separated from residual single-stranded and linear duplex DNA by hydroxylapatite chromatography. Following ligation of the nicked heteroduplexes with *E. coli* DNA ligase in the presence of ethidium bromide, the covalently closed supercoiled heteroduplex molecules were separated from the open circular form in a CsCl-ethidium bromide density gradient. The ethidium bromide was extracted by washing with *n*-butanol.

Unless otherwise indicated, the mutant strand of the heteroduplexes is fully methylated at GATC sequences, and the wild-type strand, which is derived from phage R408-infected cells, is at least 80% methylated (data not shown). Strains GM1690 and JC9239 were used to prepare unmethylated and methylated plasmid DNAs, respectively. Sensitivity or resistance to *Mbo*I and *Dpn*I was used to measure the degree of adenine methylation in DNA.

DNA hybridization (44). Heteroduplex preparations were electrophoresed in 0.8% agarose and transferred to Immobilon-N membranes (Millipore Corp., Bedford, Mass.) by capillary action. The DNA was fixed to the membrane by baking at 80°C under a vacuum for 60 min. Prehybridization was for 2 h at 65°C in 6× SSC (standard saline citrate [150 mM NaCl plus 15 mM sodium citrate])–0.5% SDS (sodium dodecyl sulfate)–5× Denhardt's solution (0.02% Ficoll 400, 0.02% bovine serum albumin, 0.2% polyvinylpyrrolidone)–2 μg of denatured salmon sperm DNA per ml. Plasmid pPY97 was used as the template to prepare ^{32}P-labelled probes by the random priming method (New England Biolabs). The membranes were incubated with the probe overnight at 55°C

TABLE 1. *E. coli* strains

Strain[a]	Relevant genotype	Source or derivation
AB259	Hfr	CGSG[b]
AB1157	Wild type[c]	CGSG
AB4206	*recA1*	CGSG
AM207	*recR252*::Tn*10* miniKan	R. G. Lloyd (28)
BW114	*uvrA6*	B. Wilkins
BW535	*nfo-1 nth-1* Δ(*xth-pnc*)*90*	B. Weiss (8)
CM5298	*polA214*(Ts) *lac*	C. Joyce
DE192	*lexA51*	D. G. Ennis
DE407	*lexA3*	D. G. Ennis
H124	*ruvB4*	R. G. Lloyd
JC7623	*recB21 recC22 sbcB15 sbcC201*	A. J. Clark
JC9239	*recF143*	A. J. Clark (15)
KL584	Hfr Δ(*lac-pro*)$_{XIII}$	K. B. Low
KM353	*recD*	K. C. Murphy (33)
KM354	*recJ*	K. C. Murphy (33)
N2445	*recO1504*::Tn*5*	R. G. Lloyd (23)
N3398	*recG258 ruvC53*	R. G. Lloyd (24)
NK5991	*proC*::Tn*5* Δ*lacU169*	N. Kleckner
MRi93	*pcnB*::Tn*10*	J. Lapilato (25)
SP254	*recN262*	R. G. Lloyd (24)
GM563	Δ(*lac-pro*)$_{XIII}$	KL584 × AB1157[d]
GM1690	*dam-16*::Kan^r	34
GM2622	pPY97	Laboratory strain
GM2801	Hfr *uvrA6 recF143 tra*::Tn*10*	Laboratory strain
GM2834	Hfr3000 Δ*lacU169*	J. Beckwith
GM4326	Δ(*lac-pro*)$_{XIII}$ (GM110)	GM563
GM4331	As GM4326 but Lac$^+$ revertant[e]	
GM4349	As GM4331 but *mutH471*::Tn*5*	35
GM4388	As GM4331 but *mutS201*::Tn*5*	35
GM4389	As GM4331 but *recA1*	Laboratory strain
GM4392	As GM4331 but *recF143*	AB259 × JC9239 × KL584
GM5808	*nfo-1 nth-1* Δ(*xth-pnc90*) (GM110)	BW535
GM5809	As GM4331 but *uvrA6 mutH471*::Tn*5*	BW114 × GM4349
GM5810	As GM4331 but *recD*	GM2834 × KM353
GM5813	As GM4331 but *recJ*	GM2834 × KM354
GM5814	As GM4331 but *recB21 recC22 sbcB15 sbcC201*	NK5991 × JC7623[f]
GM5817	As GM4331 but *ruvB4*	NK5991 × H124[f]
GM5819	As GM4331 but *recR252*::Tn*10* miniKan^r	GM2834 × AM207
GM5821	As GM4331 but *recO1504*::Tn*5*	GM2834 × N2445
GM5822	As GM4331 but *recN262*	GM2834 × SP254
GM5824	*polA214*(Ts) (GM110)	CM5289
GM5834	As GM4331 but *recB21 recC22 sbcB15 sbcC201 recF143 uvrA6*	GM2801 × GM5814

[a] All GM strains, except GM2622, GM2801, GM2834, and GM5824, are derived from AB1157.
[b] CGSG, *E. coli* Genetic Stock Culture Center, Department of Biology, Yale University, New Haven, Conn.
[c] The genotype of AB1157 is F$^-$ *thr-1 ara-14 leuB6* Δ(*gpt-proA*)*62 lacY1 tsx-33 supE44 galK2 hisG4*(Oc) *rfb-1 mgl-51 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1.*
[d] Denotes genetic cross by conjugation.
[e] The genotype of GM4331 is F$^-$ *thr-1* Δ(*lac-pro*)$_{XIII}$ *tsx-33 supE44 galK2 hisG4*(Oc) *rfb-1 mgl-51 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1.*
[f] Denotes genetic cross by P1*vir* transduction.

in hybridization buffer (6× SSC, 10 mM EDTA, 5× Denhardt's solution, 0.5% SDS, 2 μg of denatured salmon sperm DNA per ml) followed by washes with 2× SSC–0.5% SDS (5 min), 2× SSC–0.1% SDS (15 min), and 0.1× SSC–0.5% SDS (twice, 30 min) at room temperature. The radioactive bands were visualized by fluorography.

Transformations. Cells competent for transformation were prepared by the $CaCl_2$ method as described previously (4). Electroporation could not be used because it resulted in transient induction of the *mnt*::*lacZYA* fusion. Since the transformation efficiency is variable from strain to strain, the amount of heteroduplex DNA was adjusted so that approximately 100 colonies per plate were obtained. This required 25 ng for the *recBC sbcBC recF uvrA* and *recG ruvC* strains; 60 ng for the *lexA53*, *lexA3*, and *nfo xth nth* strains; 100 ng for the *pcnB* strain; 150 ng for the *recA* and *polA214*(Ts) strains; and 1 ng for all others. Cells were incubated for 30 min in L broth following heat shock treatment and plated onto MacConkey agar plates supplemented with 60 μg of ampicillin per ml. Control experiments showed that the frequency of ampicillin-resistant transformants was the same on MacConkey and broth agar. The plates were incubated at 37°C for 17 h, except for the *recA* and *recBC sbcBC recF uvrA* strains (27 h) and the *polA* bacteria (30°C, 26 h).

Screening for false-negative results. Transformant colonies that were white on MacConkey medium were patched with toothpicks onto MacConkey agar supplemented with 3.5 μg of tetracycline per ml. As shown in Fig. 1, true white colonies failed to grow on this medium, whereas bleached mixed colonies grew.

Retransformation experiments. For strains which harbored Tn*10* transposons, plasmid DNA was isolated from 25 presumptive white colonies and used to transform GM4331. True white colonies yielded only white colonies on retrans-

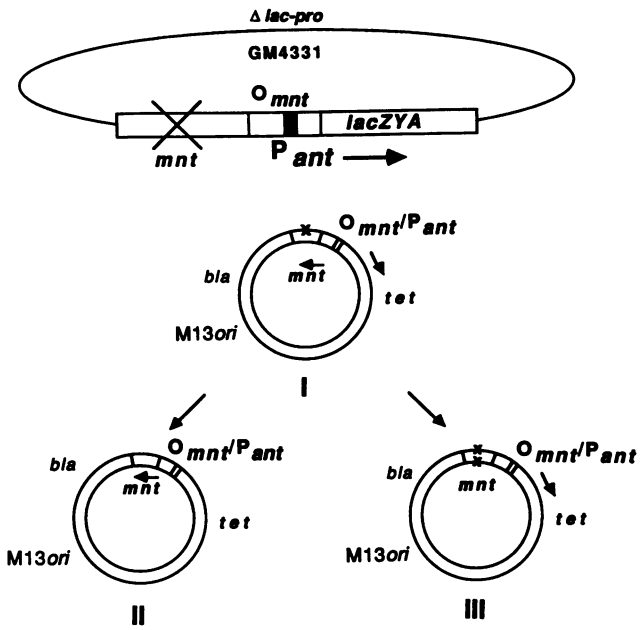

FIG. 1. The indicator system to monitor the fate of heteroduplex DNA molecules. The top part of the figure depicts the chromosome of strain GM4331 which harbors a prophage with an *mnt::lacZYA* fusion. Since no Mnt repressor is available to bind to its operator (O_{mnt}), transcription of *lacZYA* from the overlapping *ant* promoter (P_{ant}) leads to a Lac^+ phenotype. When a heteroduplex DNA molecule (I) is introduced into the cell, repair toward the wild-type genotype (II) leads to the production of active Mnt repressor which represses transcription of the chromosomal *lac* genes as well as the P_{ant}::*tet* operon fusion on the plasmid leading to the formation of colonies which are Lac^- and Tet^s. Repair of the heteroduplex molecule to the mutant configuration (III) allows transcription from P_{ant} on both plasmid and chromosome, thereby conferring a Lac^+ Tet^r phenotype on the cells in the colony. In the absence of repair of the heteroduplex DNA molecule, both plasmid species II and III are present, yielding colonies containing Lac^+ and Lac^- cells (mixed colonies). The crosses in configurations I and III represent deletions of genetic information in *mnt*. Arrows on plasmid maps denote the directions of transcription. Operator-constitutive mutations cannot be scored by the color reaction.

formation, while bleached mixed colonies yielded white and red colonies. For bacteria (*pcnB*) which were not derived from AB1157 as well as those which could not be lysogenized (*lexA51*) and those which gave an abnormal color reaction (*recBC sbcBC*), plasmid DNA was extracted from at least 25 transformant colonies and retransformed into GM4331 to score for red and/or white colonies. The retransformation method gave results identical to those based on colony color when GM4331 was transformed with the 5-base deletion heteroduplex molecules.

RESULTS

Experimental design. We wanted to determine the fate of plasmid heteroduplex molecules which contained loops of 5 bases or more after transformation into the wild type and various mutant derivatives of *E. coli*. Such heteroduplex molecules are expected to be formed by annealing a wild-type DNA strand with a mutant complementary strand deleted for a given number of nucleotides. A heteroduplex with a loop can replicate in the cell to yield equal numbers of mutant and wild-type progeny molecules (i.e., a mixed genotype). Alternatively, the heteroduplex can be repaired prior to replication to yield more product molecules of one parental genotype than the other, depending on the strandedness of repair. To discriminate easily between these two possibilities, we have devised an experimental system which allows the genotypes of plasmids in the cell to be determined on the basis of colony color. A brief description of this system has recently appeared elsewhere (35).

We have introduced mutations into the bacteriophage P22 *mnt* gene on plasmid pPY97. This plasmid carries the tetracycline resistance gene from plasmid pBR322 under the negative control of the Mnt repressor. Mutations that inactivate the *mnt* gene permit constitutive transcription from the phage *ant* promoter in this transcriptional fusion and allow the plasmid to confer tetracycline resistance to the host cell (Fig. 1). In contrast, cells containing plasmids with the wild-type *mnt* gene are tetracycline sensitive because of repression of transcription from the *ant* promoter by Mnt (49).

In addition, we constructed an *mnt::lacZYA* transcriptional fusion on a lambda prophage in a strain deleted for the chromosomal *lac* operon. Transformation of this strain (GM4331) to ampicillin resistance with a plasmid containing the wild-type *mnt* gene changed the colony color from red to white on MacConkey agar plates. Conversely, transformation of the lysogen with the plasmids, described below, deleted for part of the *mnt* gene did not change the colony color. In this assay system, therefore, ampicillin-resistant colonies containing mutant *mnt* plasmids are red and tetracycline resistant, colonies containing the wild-type *mnt* plasmid are white and tetracycline sensitive, and colonies arising from cells containing both mutant and wild-type plasmids (resulting from transformation with heteroduplex DNA) show both white and red (mixed) coloration (see below).

In practice, we found that some of the mixed class resulting from transformation with heteroduplex DNA could not easily be distinguished from white colonies because of spontaneous bleaching of the red color. To estimate the percentage of false white colonies, we took advantage of the differential sensitivities to tetracycline of white and mixed colonies. True white colonies are tetracycline sensitive, whereas mixed colonies show a range of resistance concentrations, with a threshold at 3.0 μg/ml. At least 104 white colonies from each transformation experiment described below were patched onto medium containing 3.5 μg of tetracycline per ml to estimate the percentage of false white colonies.

We also tested the validity of the assay system by isolating plasmid DNA from 20 white, 20 red, and 20 mixed colonies obtained after transformation with heteroduplex DNA and by retransforming the indicator strain, GM4331. Plasmid DNA from the red or white colonies yielded only red or white colonies, respectively, on retransformation. Plasmid DNA from the mixed colonies yielded both red and white colonies in various proportions, ranging from 88% red and 12% white to 1% red and 99% white.

Construction of mutants. Mutations deleting 7 or 9 nucleotides were introduced by oligonucleotide-directed mutagenesis into the *mnt* repressor gene to produce nonfunctional repressor proteins. The mutations were targeted to the His-6 residue of the repressor, which has been shown by both genetic (49) and biochemical (19, 20) means to be in direct contact with operator DNA (Fig. 2). The mutations deleting 5 nucleotides in *mnt* or 1 base in the Mnt operator have been described elsewhere (5, 35).

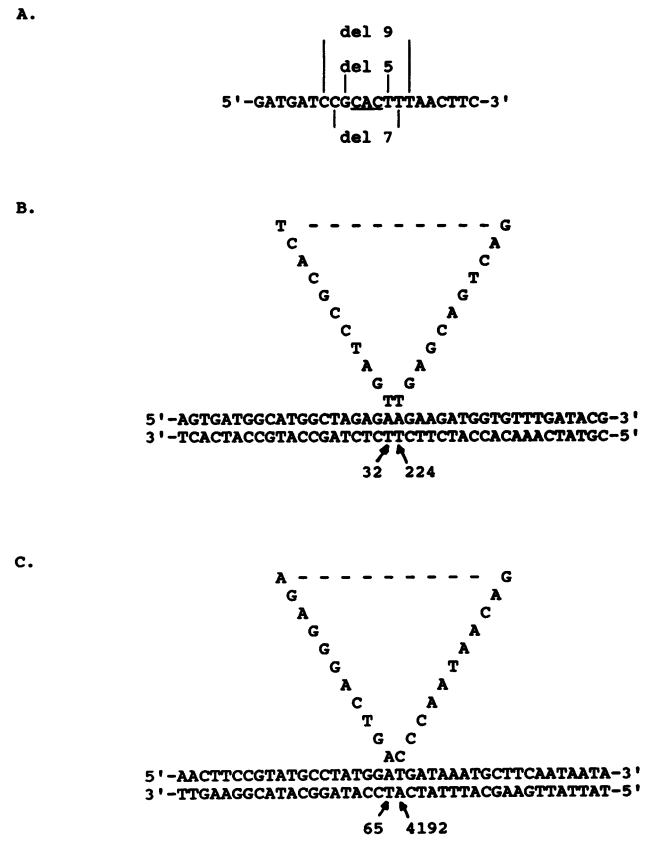

FIG. 2. (A) The DNA sequence of part of the *mnt* gene, together with the locations of the deletion (del) mutations, is shown. The underlined bases constitute the His-6 codon. (B) DNA sequence at the base of the 192-base loop. The lower strand is derived from a mutant plasmid in which nucleotides 33 to 223 have been deleted. The upper strand is wild type, thereby producing a multibase loop in the heteroduplex region upon annealing of the two strands. The V shape indicates that there is little base-pairing capability between the nucleotides at the 5′ and 3′ ends of the looped-out region. The dashes indicate the remainder of the loop. (C) The same as for panel B but for the 410-bp deletion. Nucleotide sequence numbering is that of Carraway et al. (5).

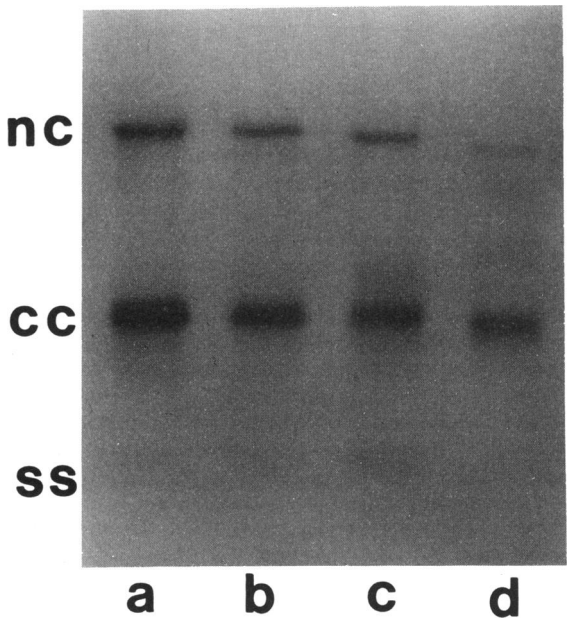

FIG. 3. Southern blot analysis of the heteroduplex DNA preparations. The DNA molecules were separated in an agarose gel, blotted to a nylon membrane, and probed with wild-type plasmid DNA labelled with ^{32}P. The membranes were visualized by autoradiography. The figure shows heteroduplex DNA preparations with multibase loops of 7 (lane a), 9 (lane b), 192 (lane c), and 410 nucleotides (lane d). The positions of nicked circular (nc), covalently closed supercoiled (cc), and single-stranded (ss) molecules are also shown.

The 192- and 410-nucleotide deletions affecting *mnt* were isolated as mutations produced by DNA polymerase III holoenzyme replication of template DNA in vitro in the absence of single-stranded binding protein (4). The 192-nucleotide deletion removes about two-thirds of the *mnt* gene, while the 410-nucleotide deletion removes part of the *mnt* gene and the intergenic region between *mnt* and the *bla* (β-lactamase) genes. Figure 2B and C show the sequence of the DNA flanking each of the deletions. Figure 2 also compares the sequences of the terminal 11 nucleotides at the extremities of the single-stranded regions in the heteroduplex loops and shows that only one complementary (Watson-Crick) base pair is predicted to be formed.

Purity of heteroduplex preparations. The covalently closed supercoiled heteroduplex molecules were prepared as described in Materials and Methods. At the end of the studies to be described, a sample of each heteroduplex was subjected to electrophoresis in an agarose gel, blotted to a nylon membrane, and probed with randomly primed wild-type *mnt* plasmid DNA. The results in Fig. 3 show that between 75 and 90% of the heteroduplex molecules were present in the supercoiled form. The major additional species was the nicked circular form. The supercoiled, nicked circular and single-stranded forms from each heteroduplex preparation shown in Fig. 3 were isolated from the agarose gel and used to transform the indicator strain, GM4331. Of the total transformants, the covalently closed supercoiled molecules were responsible for more than 90% (data not shown). No transformants were obtained from the band in Fig. 3 migrating at the position of single-stranded DNA. The nicked circular form yielded the balance of the transformants but gave an identical distribution of red, white, and mixed colonies as the supercoiled plasmid DNA shown in Table 2. The heteroduplex DNA with the 5-base loop gave a distribution of nicked, supercoiled, and single-stranded DNA similar to the distributions shown in Fig. 3 (data not shown).

Transformation of the wild-type strain with heteroduplex DNA containing a 5-base loop. Strain GM4331 was transformed with symmetrically methylated heteroduplex DNA containing a 5-base loop by selection for ampicillin-resistant transformants on MacConkey agar. Table 2 shows that about 90% of the GM4331 transformants had a mixed genotype, consistent with replication of the heteroduplex DNA without detectable repair. DNA sequence analysis of the *mnt* region from plasmid DNA of some red colonies showed no alteration other than the 5-base deletion (data not shown).

We have shown previously that heteroduplex molecules with a 5-base loop are not substrates for *dam*-directed repair (35). Heteroduplex molecules which were fully methylated or completely unmethylated on both strands or in which only one of the two strands was methylated yielded about 90% mixed colonies after transformation of wild-type or *mutS*

TABLE 2. Distribution of colony color after transformation of the wild-type strain with heteroduplex DNA[a]

Loop size in bases	Percent			No. of colonies scored
	Red	White	Mixed	
1	98	1	1	262
5	4	5	91	1,311
7	17	9	74	571
9	23	8	69	685
192	25	18	57	541
410	25	15	60	435

[a] Competent cells of strain GM4331 were mixed with 1 ng of covalently closed, supercoiled, symmetrically methylated heteroduplex DNA. The transformation mixture was plated on MacConkey agar with 60 μg of ampicillin per ml, 30 min after heat shock. The plates were scored after incubation for 17 h at 37°C. The 1-base loop heteroduplex DNA was methylated only on the mutant strand, thus directing repair to the wild-type strand (35). In a *mutS* strain, the same heteroduplex yielded 4% red, 4% white, and 92% mixed colonies.

TABLE 3. Distribution of colony color after transformation of *polA214*(Ts) cells with heteroduplex DNA containing a loop of 7 bases in the *mnt* gene[a]

Temp (°C)	%			No. of colonies scored
	Red	White	Mixed	
30	21	12	68	778
42	16	7	77	693

[a] Competent cells of the *polA214*(Ts) strain were mixed with 150 ng of supercoiled heteroduplex DNA. After heat shock, the transformation mixture was incubated at either 30 or 42°C for 30 min before being plated on MacConkey agar with 60 μg of ampicillin per ml. The plates were scored after 26 h of incubation at 30°C.

strains (35). Furthermore, MutS protein in vitro did not bind to heteroduplex regions containing a 5-base loop (35) or to molecules with 7- or 9-base loops (3a).

As a positive control, a hemimethylated heteroduplex molecule with a 1-base deletion in *mnt* is efficiently repaired in the direction predicted by the methylation asymmetry (35) (Table 2). The same heteroduplex molecule was not detectable repaired in a *mutS* recipient (see footnote *a* to Table 2).

Transformation of the wild-type strain with heteroduplexes containing 7-, 9-, 192-, and 410-base loops. In contrast to the results for the 5-base deletion heteroduplex DNA, the heteroduplexes with larger loops yielded mixed transformants at a lower frequency, ranging from 57 to 74% (Table 2). This decrease could be due to impure heteroduplex preparations, DNA repair, strand loss, or aberrant segregation of plasmid molecules. It is unlikely that this decrease is due to contaminants in the heteroduplex DNA preparations (see above). The *mnt* region in plasmids extracted from several red colonies from each transformation was sequenced, but no alteration other than the original mutation was detected. This result excludes the possibility of extensive processing of the heteroduplex DNA.

It is also possible that the results reflect a slow DNA repair process and that the 30-min incubation time in the transformation procedure was sufficient to allow repair of only a fraction of the molecules. We could not prolong the time of incubation of the cells after the heat-shock step in the transformation procedure because of replication of the plasmid and cell division. We will return to the question of slow repair in a subsequent section.

Transformation of repair- and recombination-deficient mutant strains. If the reduction in mixed colonies of the 7- to 410-base deletion-insertion heteroduplexes is due to repair, then the proportion of the mixed-genotype class should increase in a strain deficient in such repair. The following mutant strains were tested with all of the heteroduplex DNA molecules: *recA*, *recBC sbcBC*, *recD*, *recF*, *recJ*, *recN*, *recO*, *recR*, *ruvB*, *uvrA*, *lexA51*(Def), *lexA3*(Ind), *recG ruvC*, *recBC sbcBC recF uvrA*, *mutS*, and *nfo xth nth*. In none of these strains did the proportion of mixed colonies increase significantly compared with those shown in Table 2 for the wild type (data not shown).

The result with the *mutS* strain rules out a role for *dam*-directed mismatch repair. The *nfo xth nth* result indicates that exonuclease III and endonucleases III and IV do not affect segregation of the heteroduplex molecules. The loop is not recognized by the UvrABC enzyme and is not processed by the SOS repair system, on the basis of the results with the *recA* and *lexA* mutants.

We considered that the loop structure might resemble a recombination intermediate which could be processed by recombination enzymes. The lack of any significant effects in *rec* mutants makes this unlikely, as does the result with the *ruv* mutants (which may be involved in resolving Holliday structures [7, 11, 17, 23]).

There was no significant change in a multiple-mutant strain (*recBC sbcBC recF uvrA*) deficient in the RecBCD and RecF recombination systems. The RecF protein is an absolute requirement for plasmid recombination (18), thus making it unlikely that plasmid recombination contributes to the reduction in mixed colonies.

Transformation of a *polA*(Ts) strain. To test the possibility that there was a low rate of repair which was limited in its action by plasmid replication, we incubated *polA*(Ts)-transformed cells at permissive and nonpermissive temperatures. In ColE1-derived plasmids, initiation of DNA replication occurs by cleavage of an RNA-DNA hybrid, which is followed by extension of the RNA primer by DNA polymerase I. After a few hundred nucleotides, DNA polymerase III holoenzyme takes over to complete the process (16, 45). In a *polA* strain at the nonpermissive temperature, the heteroduplex DNA will be unable to replicate, thus giving the repair system, if it exists, more time to act prior to replication. The results in Table 3 show that the frequency of mixed colonies was not significantly different at the nonpermissive temperature than it was at the permissive temperature. The distribution of colony types was unchanged even after 60 min of incubation at the nonpermissive temperature (data not shown).

The frequency of ampicillin-resistant transformants at the two temperatures, however, was the same. This makes it less likely that DNA polymerase I itself was required as part of a repair process, since attempted repair in the absence of this enzyme would lead to DNA chain breaks and a reduction in plasmid survival.

Transformation of a *pcnB* mutant strain. To test the possibility that plasmid copy number could influence the yield of mixed colonies, we have utilized a *pcnB* mutant strain. In such strains, the copy number of ColE1 plasmids is reduced (25) to three to four per cell under the conditions used in these experiments (data not shown). The *pcnB* strain was transformed with the various heteroduplexes, but the resulting distributions of transformant classes did not differ significantly from those shown in Table 2 for the wild type.

Segregation of two plasmid markers. The data described

TABLE 4. Genetic constitution of red and white colonies after transformation with heteroduplex DNA containing 7- and 514-base loops on one strand[a]

Colony type	No. with *AvaI*$^+$	No. with *AvaI*$^-$
Red (n = 28)	0	28
White (n = 23)	23	0

[a] Wild-type cells (GM4331) were transformed with heteroduplex DNA as described in footnote *a* to Table 2. The color distribution among 612 transformant colonies was 16% red, 6% white, and 78% mixed colonies. Plasmid DNA was extracted from red and white colonies, purified, and digested with *Ava*I. The presence of two bands after digestion indicates that the 514-bp M13*ori* is present (*AvaI*$^+$).

above indicate that after transformation of cells with heteroduplexes containing loops of 7 bases or more, only 57 to 74% of mixed colonies are recovered. This observation is based, however, on the behavior of a single genetic marker, *mnt*. We decided to monitor two markers on each heteroduplex strand to see whether they would segregate together, suggesting strand loss, or if they would reassort, suggesting repair.

In addition to the heterologous sequence for *mnt*, we introduced a second heterology by deleting the 514-bp M13 origin of replication (*ori*) from one of the heteroduplex strands. The configuration of the heteroduplex was *mnt*$^+$ and M13*ori*$^+$ on one strand, and the 7-base deletion in *mnt* without the M13*ori* region was in the complementary strand. The shorter distance separating the two markers is 1,369 nucleotides.

The wild-type strain, GM4331, was transformed with the heteroduplex DNA and ampicillin-resistant transformants selected on MacConkey agar. The distribution of red, white, and mixed colonies was similar to that of the 7-base deletion-insertion heteroduplex in Table 2. The presence of the unselected M13*ori* marker in the red and white colonies was monitored by sensitivity to *Ava*I digestion in plasmid DNA isolated from such colonies. The presence of the M13*ori* results in the formation of two *Ava*I DNA fragments, but only one is formed in its absence. The data in Table 4 indicate that the two markers (*mnt* and M13*ori*) segregated together in the red and white colonies. This suggests that the red and white colonies arise by the random loss of one or the other parental strand.

Since the M13*ori* cosegregates with the wild-type marker, it also cannot be subject to repair. That is, the data described above show that single-stranded regions of 514 bases are not repaired in wild-type *E. coli*.

That the parental configuration is maintained in the red and white colonies makes it less likely that their appearance is due to DNA repair, since conversion of one marker would be expected to occur at a higher frequency than that of two unlinked markers.

Corepair of loops and mismatches. Since the results described above suggested that there was no multibase loop-specific repair system in *E. coli*, we wished to test whether a mismatch near a loop could provoke the repair of both (corepair). We chose a mutation in the Mnt operator which was isolated from a *dam* mutant as a tetracycline-resistant derivative and is deleted for the thymine residue at position −43 (5). A heteroduplex containing a methylated wild-type strand and a complementary unmethylated strand deleted at position −43 was used to transform wild-type and *mutS* cells to ampicillin resistance. As shown in Table 5, 97% of the

TABLE 5. Repair of a mismatch (A/−) in the Mnt operator and corepair of a 514-base loop 1,448 nucleotides upstream[a]

Strain	%		No. of Tet^s colonies with	
	Tet^s	Tet^r	*AvaI*$^-$	*AvaI*$^+$
Heteroduplex I (A/−)				
GM4331 (wild type)	97	3		
GM4338 (*mutS*)	28	72		
Heteroduplex II (A/− and −/loop)				
GM4331 (wild type, [n = 22])	94	6	21	1
GM4338 (*mutS*)	24	76		

[a] Heteroduplex I contains a wild-type methylated strand and an unmethylated complementary strand with a deletion at position −43 in the Mnt operator. Heteroduplex II contains a methylated strand with a wild-type Mnt operator that lacks an M13*ori* region and a complementary unmethylated strand with an M13*ori* but that is deleted for the thymine at −43 in the Mnt operator. The numbers of ampicillin-resistant transformants analyzed from strains GM4331 and GM4338 were 72 and 68, respectively. Plasmid DNA was isolated from the Tet^s transformants and digested with *Ava*I. Plasmids containing the M13*ori* were cut twice (*AvaI*$^+$), those without it were cut once (*AvaI*$^-$).

molecules were corrected to the wild-type (Tet^s) configuration. In contrast, most of the transformants from the *mutS* strain were tetracycline resistant, indicating either a mixed genotype or the parental operator-constitutive mutation. Retransformation experiments indicated that they had a mixed genotype, since both wild-type and mutant plasmids were recovered (data not shown). These data indicate that an unpaired adenine in a heteroduplex is efficiently repaired by the *dam*-directed system.

We constructed a heteroduplex which contained the 514-base M13*ori* loop on the unmethylated strand separated by 1,448 nucleotides from the unpaired adenine in the Mnt operator on the complementary methylated strand. The results in Table 5 show that in the wild-type host, 94% of the mismatches in the Mnt operator were repaired to the wild-type configuration (Tet^s). In 21 of 22 of these wild-type transformants, there was corepair, since the M13 *ori* region was no longer present, as determined by sensitivity to *Ava*I cleavage. This indicates that corepair is very efficient (95%) over a distance of 1,400 bases. We assume the repair system chooses the shorter (1,400 bases) rather than the longer (4,000 bases) path, although we have no direct evidence for this point.

Table 5 also shows that in a *mutS* strain, most of the transformants were tetracycline resistant, consistent with severely reduced repair of the mismatch in the Mnt operator. Plasmids isolated from these colonies yielded both parental genotypes after retransformation, indicating that the tetracycline-resistant colonies had a mixed genotype (data not shown). Digestion with *Ava*I of selected plasmid DNAs confirmed this conclusion (data not shown).

We also tested for corepair of the mismatch in the Mnt operator and the 410-base loop in *mnt*, which are separated by 107 nucleotides. The methylated strand of the heteroduplex was deleted for the 410 bases (which removes the only *Eco*RI site in the plasmid), and the complementary unmethylated strand was deleted for the thymine in the Mnt operator. Table 6 shows that 85% of the transformed cells yielded red colonies, indicating efficient removal of the 410-base loop from the unmethylated strand in the wild-type cell. This was confirmed by the absence of an *Eco*RI site in plasmids isolated from 23 of 24 red colonies. In contrast, in

TABLE 6. Corepair of a mismatch (A/−) in the Mnt operator and a 410-base loop 107 nucleotides upstream[a]

Strain	%		
	Red	White	Mixed
GM4331 (wild type)	85	0	15
GM4338 (*mutS*)	11	0	89

[a] The heteroduplex contains a methylated strand that is deleted for 410 bases of *mnt* DNA but wild type for the Mnt operator region. The complementary unmethylated strand is wild type for the *mnt* gene but deleted for base −43 in the operator. The numbers of ampicillin-resistant transformants analyzed from strains GM4331 and GM4338 were 126 and 220, respectively.

the *mutS* strain most of the transformants were tetracycline resistant, and retransformation of plasmids isolated from these colonies indicated a mixed genotype indicative of reduced repair of the loop.

In the experiment described above, we do not know the extent of repair of the mismatch in the Mnt operator, since we have no easy way to score for the presence or absence of the operator mutation. We assume, on the basis of the results from Table 5, that the repair efficiency must be greater than 85%.

DISCUSSION

Our results indicate that there is no system in *E. coli* specific for the repair of loops larger than 4 bases in heteroduplex DNA. Our results extend the finding of Raposa and Fox (38), who showed that a phage lambda heteroduplex DNA molecule with a 700-base loop can replicate without apparent repair. Multibase loops, however, can be removed by *dam*-directed mismatch repair if a repairable mismatch is in the vicinity. Although we have tested only the 410- and 514-base loops, it is reasonable to assume that smaller loops will also be corepaired. These results highlight the versatility of the *dam*-directed system in its ability not only to repair base-base mismatches and insertions-deletions of up to 4 nucleotides but also indirectly to include loops of at least 514 bases by corepair.

We were surprised that multibase loops in heteroduplex DNA are stable in cells. One reason may be that proteins such as Ssb bind to and protect the loop from attack by nucleases. If they exist, such proteins are presumably displaced during replication and *dam*-directed mismatch repair. Although Ssb protein is expected to bind to large loops (e.g., greater than 100 bases), other proteins might be expected to bind to small (5- to 7-base) loops. We have not tested an *ssb* mutant in our system, since it is essential for viability.

We have shown previously that the frequency of spontaneous mutation in the Mnt operator is correlated with the state of plasmid multimerization (3). Mutation frequency increased approximately 10- and 100-fold in dimers and trimers, respectively, relative to monomeric plasmids. We suggested that in multimeric plasmids, pairing out of register could stimulate the formation of multibase loops which, because of their single-stranded nature, are more prone to mutations. Our current findings on the stability of loops lend credence to this idea.

Our results appear to be at variance with those of Dohet et al. (10), who found that phage lambda DNA heteroduplexes with an IS*1* insertion (768 bp) on one strand appeared to be repaired in wild-type, *dam*-directed mismatch repair-defective, *uvrA*, *recA*, *recBC*, and *recF* bacteria. Unlike the case with our results, however, strand loss was not the explanation, since two markers on the same strand did not segregate together. Insertion elements which are able to form stable stem-loop structures may be processed very differently from those used in the present work. For example, Raposa and Fox (38) demonstrated that transformation of *E. coli* cells with phage lambda DNA heteroduplexes containing a Tn*10* in one strand led to loss of the transposon in the progeny.

Our results also appear to be at variance with those of Fishel and Kolodner (13), who found that a 10-base *Xho*I linker in one strand of a plasmid heteroduplex molecule is repaired in *E. coli*. Repair was reduced in *recF*, *recJ*, and *ssb* mutant strains. The reduction of repair in the *rec* mutants was about 50-fold relative to that of the wild type. To reconcile the difference between their results and ours, we propose that the *Xho*I linker sequence adopts a configuration suitable for repair, whereas the loops that we have studied do not. This configuration may be palindromic, as would be the IS*1* and Tn*10* constructs that are referred to above.

Heteroduplex DNA containing a mismatch and a nearby loop can be formed during genetic recombination in crosses with parental strains each containing one of the mutations. Corepair could result in an excess of one of the parental genotypes in the progeny. For example, the findings of Pasta et al. (36) that long deletions on a donor fragment are converted to wild type if the recipient pneumococcus contains a linked point mutation appear to support this idea.

As noted in the Introduction, we found previously that deletions were recovered at high frequency after replication of the *mnt* region in vitro by DNA polymerase III in the absence of Ssb protein (4). These deletions must have arisen during in vitro replication presumably because of the formation of secondary structures allowing slippage of the polymerase on the template strand to form heteroduplex DNA with loops. This suggests that Ssb protein may be required to prevent this type of mutation.

From the results presented in this paper, we conclude that multibase loops of 5 to 514 nucleotides in DNA molecules are not efficiently repaired in *E. coli*. Such loops in unmethylated DNA strands can be removed, however, by the *dam*-directed system as a consequence of a corepair event.

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