EFFECTS OF HIGH LEVELS OF DNA ADENINE METHYLATION ON METHYL-DIRECTED MISMATCH REPAIR IN *ESCHERICHIA COLI*

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ABSTRACT

Two methods were used in an attempt to increase the efficiency and strand selectivity of methyl-directed mismatch repair of bacteriophage λ heteroduplexes in *E. coli*. Previous studies of such repair used λ DNA that was only partially methylated as the source of methylated chains. Also, transfection was carried out in methylating strains. Either of these factors might have been responsible for the incompleteness of the strand selectivity observed previously. In the first approach to increasing strand selectivity, heteroduplexes were transfected into a host deficient in methylation, but no changes in repair frequencies were observed. In the second approach, heteroduplexes were prepared using DNA that had been highly methylated *in vitro* with purified DNA adenine methylase as the source of methylated chains. In heteroduplexes having a repairable *cI*/+ mismatch, strand selectivity was indeed enhanced. In heteroduplexes with one chain highly methylated and the complementary chain unmethylated, the frequency of repair on the unmethylated chain increased to nearly 100%. Heteroduplexes with both chains highly methylated were not repaired at a detectable frequency. Thus, chains highly methylated by DNA adenine methylase were refractory to mismatch repair by this system, regardless of the methylation of the complementary chain. These results support the hypothesis that methyl-directed mismatch repair acts to correct errors of replication, thus lowering the mutation rate.

THE principal modified base in the DNA of *Escherichia coli* is 6-methylaminopurine, which is formed by the postreplicative methylation of adenine at GATC sequences. The reaction is carried out by the enzyme DNA adenine methylase, coded by the *E. coli dam* gene (MARINUS and MORRIS 1973; LACKS and GREENBERG 1977; GEIER and MODRICH 1979).

Studies of DNA mismatch repair in artificially constructed heteroduplexes of bacteriophage λ infecting *E. coli* have shown that the presence of 6-methylaminopurine at GATC sites strongly influences the strand specificity of repair.

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If one chain of the heteroduplex is methylated and the other is not, mismatch repair is favored on the unmethylated chain (RADMAN, WAGNER, GLICKMAN and MESELSON 1980; M. RYKOWSKI and M. MESELSON, unpublished results). This finding supports the hypothesis that, in *E. coli*, errors of DNA replication are eliminated by mismatch repair occurring preferentially on the newly synthesized polynucleotide chain. According to this hypothesis (WAGNER and MESELSON 1976), the necessary discrimination between old and new chains is made possible by the transient undermethylation of the latter. Such methyl-directed mismatch repair would presumably be advantageous in reducing the frequency of mutation.

For all repairable mismatches examined to date, methylation of one chain of the λ heteroduplex influences the strand specificity of repair in the predicted direction. However, considerable repair in the opposite direction is also observed. Although we realized that transfecting λ heteroduplexes may provide an imperfect model for mismatch repair events at an *E. coli* replication fork, we nevertheless sought conditions that increase the strand selectivity of repair in this experimental system.

Since previous transfections had been carried out in *dam*$^+$ bacteria, we first asked whether methylation of the heteroduplexes occurring before repair is sufficient to decrease the discrimination of the repair system. We performed transfections in a host lacking the methylase, but no alteration in frequency or strand selectivity of repair was noted. Apparently, under these conditions, repair occurs before methylation can proceed far enough to affect the outcome.

When we examined a second possible cause of incomplete strand discrimination, we observed, as others have (LACKS and GREENBERG 1977), that methylation at GATC sequences in λ DNA isolated from ordinary phage lysates is much less complete than it is in *E. coli* DNA. λ DNA is presumably encapsidated before methylation can proceed to a high level. Such phage had been used as the source of methylated chains in constructing all heteroduplexes previously examined. Therefore, we conducted experiments using as the source of methylated chains λ DNA with GATC sequences highly methylated *in vitro* with purified *dam* methylase (GEIER and MODRICH 1979). When we studied repair of a particular mismatch that had previously been tested in heteroduplexes containing incompletely methylated chains, two striking effects were observed. First, in heteroduplexes with one chain highly methylated and its complement not methylated, the frequency of repair on the methylated chain was markedly decreased and repair on the unmethylated chain was enhanced, increasing nearly 100%. Second, in contrast to the behavior of the heteroduplex with both chains only partially methylated, the heteroduplex with both chains highly methylated was refractory to repair. We conclude that (1) methyl-directed mismatch repair can eliminate certain replication errors with high efficiency, and (2) this mismatch repair system is unable to operate or operates poorly on fully methylated DNA molecules.

MATERIALS AND METHODS

The relevant characteristics of bacteria and phage are listed in Table 1. The growth and purification of phage stocks (WILDENBERG and MESELSON 1975) and the isolation, strand separation

TABLE 1

Bacteria and phage

Strain or genotype	Relevant characteristics	Reference
	E. coli k12	
C600	Wild type for methylation and mismatch repair	APPLEYARD 1954
152	Wild type for methylation and mismatch repair, *recA*	WILDENBERG and MESELSON 1975
GM119	*dam-3 dcm-6*	M. G. MARINUS, personal communication
GM33	*dam-3*	MARINUS and MORRIS 1974
ES-568	*mutL13*	LIEBERFARB and BRYSON 1970
	Lambda	
imm434	Immunity 434	KAISER and JACOB 1957
b2	Partial deletion of *att*	KELLENBERGER, ZICHICHI, and WEIGLE 1960
bio69	*int* through *exo* deleted	MANLY, SINGER and RADDING 1969
bio256	*int* through *cIII* deleted	WILDENBERG and MESELSON 1975
mi20	Minute plaque	WILDENBERG and MESELSON 1975
cI27	Clear plaque, isolated from λ*b2 bio69*, designated *c* in the text	RYKOWSKI and MESELSON 1983

and reannealing of DNA (MESELSON and YUAN 1969) were done as described previously, except that phenol was replaced with phenol/chloroform (1:1) and the D_2O-H_2O gradient was omitted. Stocks of λ*b2 bio69* and λ*b2 bio69 c* were prepared on *E. coli* strain C600 for partially methylated DNA and on GM119 or GM33 for unmethylated DNA. Highly methylated DNA was obtained by methylation *in vitro* of DNA from phage grown on C600, using DNA adenine methylase purified from an overproducing strain harboring the plasmid pGG503 (GEIER and MODRICH 1979; HERMAN and MODRICH 1981). Contamination of individual chain preparations by the complementary chain was always less than 5%, as measured by transfection titers after self-annealing and also as estimated by agarose gel electrophoresis. Transfections were performed as described (WILDENBERG and MESELSON 1975), using λ*imm434 b2 bio256* helper phage. Infected cells were plated well before lysis on 152(λ*imm434 red14 mi20*), and plaques were scored by inspection as clear, mixed or turbid. Control platings of an equal mixture of λ*b2 bio69* and λ*b2 bio69 c* showed that, at the plating densities employed, plaque overlap causes a negligible number of plaques to be scored as mixed. Gel electrophoresis was carried out using either horizontal 0.5% Seakem agarose gels in 40 mM Tris-acetate, pH 7.8, 5 mM sodium acetate, 1 mM EDTA or vertical 5% Biorad acrylamide gels in 90 mM, pH 8.3, Tris-borate, 2.5 mM EDTA. Gels were stained with ethidium bromide and photographed through a Wratten 23A gelatin filter with Kodak Tri-X film.

RESULTS AND DISCUSSION

All heteroduplexes were constructed from separated chains of λ*b2 bio69* and λ*b2 bio69 c*. The *cI* allele is a spontaneous mutant of λ*b2 bio69*, used in order to have stocks that are isogenic except at the site of the mutation.

Heteroduplexes with partially methylated chains transfecting dam *and* dam$^+$ *cells*: We constructed all eight heteroduplexes involving the four combinations of partially methylated and unmethylated chains (me^+/me^+, me^-/me^-, me^+/me^- and me^-/me^+) with each of the two different mismatches (*c* on the light chain, + on the heavy chain and *vice versa*). *E. coli* strain GM119, which lacks DNA adenine methylase, was transfected with these heteroduplexes and plated before lysis. For transfection with each type of heteroduplex, the percentage of

resulting plaques that were clear (only *c* phages present), mixed (both *c* and + phages present) and turbid (only + phages present) are given in Table 2, part A.

Heteroduplex I (first four lines of the Table) is efficiently repaired regardless of the combination of unmethylated and partially methylated chains. This is shown by the low frequency of mixed plaques, 4% or less. However, when only the light chain is methylated (line 3), most of the repair is on the heavy chain. When only the heavy chain is methylated (line 4), the asymmetry is reversed. This reversal, in which repair is favored on the unmethylated chain, has been found to occur with all nine repairable mismatches thus far examined and is the principal evidence for methyl-directed mismatch repair (RADMAN *et al.* 1980; M. RYKOWSKI and M. MESELSON, unpublished results; J. J. PETERSON and M. MESELSON, unpublished results).

Heteroduplex II, with + on the light chain and *c* on the heavy chain (last four lines of the Table) involves mismatched bases complementary to those in heteroduplex I and is, therefore, chemically distinct. Heteroduplex II is refractory to repair. In this heteroduplex, when one chain is methylated and the complementary chain is unmethylated, little or no effect on the distribution of plaque types is seen when the asymmetry of methylation is reversed (lines 7 and 8). Moreover, in the transfection system that we employ, 60–70% of the bursts yield progeny of only one of the two chains of an infecting heteroduplex due to the random loss of one or the other chain or its progeny. Such strand loss occurs after the time for repair has passed and is independent of genotype (WAGNER and MESELSON 1976). Because of strand loss, the upper limit for the frequency of mixed plaques, in the absence of repair, is about 35%. Therefore, the production of nearly this frequency of mixed plaques by transfections with heteroduplex II provides further evidence that it is repaired at a much lower frequency than heteroduplex I. The low repair frequency of heteroduplex II and the accompanying lack of methyl-directed strand selectivity provide a useful control (lines 7 and 8). This lack of effect of methylation demonstrates that the methylation state of a chain *per se* does not influence its contribution to the progeny of a transfection. Only through its effect on mismatch repair does methylation affect the genotype of progeny phage.

The pattern of heteroduplex repair in the methylase-deficient host GM119 may be compared with that previously found by M. RYKOWSKI and M. MESELSON (unpublished results) in the *dam*$^+$ host *E. coli* 152 (Table 2, part B). No significant difference is seen. In particular, the strand selectivity of repair in heteroduplex I is equally incomplete in the two strains. The substantial frequency of repair on the methylated chain, in both *dam*$^+$ and *dam* hosts, is especially evident in the heteroduplex with the heavy chain methylated and the light chain unmethylated (line 4). Although the total frequency of repair is high, as indicated by the low percentage of mixed plaques, the marker on the unmethylated chain (*c*) fails to be corrected in about 25% of the heteroduplexes. We conclude that no enhancement of strand selectivity is achieved by carrying out the transfection in the absence of methylase. Apparently, in *dam*$^+$ bacteria, methylation of transfecting heteroduplexes is too slow to influence the strand specificity of repair.

TABLE 2

Lack of influence of host methylase on mismatch repair of transfecting heteroduplexes

Heteroduplex[a]		Plaques							
		A *dam* host (GM119)				B *dam*$^+$ host (152)[b]			
		c	Mixed	+	*n*[c]	*c*	Mixed	+	*n*
I	me$^+$/me$^+$	66%	1%	33%	436	53%	3%	44%	444
c / *ℓ*	me$^-$/me$^-$	78	3	18	318	73	4	23	331
h	me$^+$/me$^-$	93	0	7	348	90	3	7	441
+	me$^-$/me$^+$	26	1	73	321	22	4	74	417
II	me$^+$/me$^+$	42	24	34	247	33	31	36	335
+ / *ℓ*	me$^-$/me$^-$	30	28	42	74	30	30	40	262
h	me$^+$/me$^-$	37	22	40	278	35	23	42	519
c	me$^-$/me$^+$	35	33	31	308	33	29	38	404

[a] Heteroduplexes are drawn with the light chain (*ℓ*) on top and the heavy chain (*h*) below. With respect to the conventional genetic map of lambda, the 5′ → 3′ direction is rightward on the light chain and leftward on the heavy chain.

[b] Data of RYKOWSKI and MESELSON 1983.

[c] The number (*n*) of plaques scored.

We confirmed that λ DNA from phages propagated on *dam* bacteria is indeed unmethylated at GATC sequences by digesting it with restriction enzyme *Mbo*I, which cleaves only if the adenines on both chains of this symmetrical sequence are unmethylated (GELINAS, MYERS and ROBERTS 1977). The completeness of the digestion (Figure 1, lane 4) contrasts with the limited extent of digestion of λ DNA from the *dam*$^+$ strain C600 (Figure 1, lane 3). Control digestions with *Hae*III, which cleaves at GGCC (NATHANS and SMITH 1975), show that both DNAs are equally susceptible to digestion by an enzyme unaffected by adenine methylation (Figure 1, lanes 1 and 2). The observed *Mbo*I digestion of λ DNA from *dam*$^+$ bacteria shows that a substantial proportion of its GATC sequences are unmethylated.

Heteroduplexes with highly methylated chains: To obtain highly methylated DNA, we further methylated λ*b2 bio69* and λ*b2 bio69 c* DNA from strain C600 with purified DNA adenine methylase. This resulted in preparations completely resistant to digestion by *Mbo*I (Figure 2, lane 3). The highly methylated DNAs were subjected to strand separation and annealing with separated unmethylated DNA chains to produce the eight possible heteroduplexes. The unmethylated chains were obtained from λ propagated on *E. coli* GM33. The lack of adenine methylation at GATC sequences was verified by restriction analysis with *Mbo*I (results not shown). The integrity and purity of the separated DNA chains is shown in the gel electrophoretic analysis of Figure 3 and is typical of our separated chain preparations. Strain GM33 lacks DNA adenine methylase but, unlike strain GM119, which was used for preparing unmethylated λ DNA used in previous experiments, it does not lack DNA cytosine methylase. Therefore, the sole difference that should exist between unmethylated and highly meth-

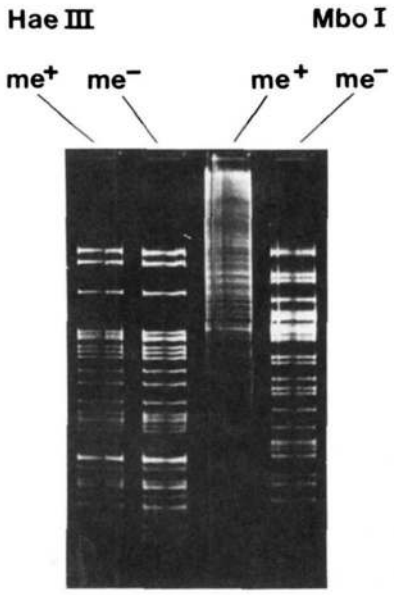

FIGURE 1.—Restriction of partially methylated (me$^+$) and unmethylated (me$^-$) DNA of λ*b2 bio69* c. DNA was digested with *Hae*III (lanes 1 and 2) and *Mbo*I (lanes 3 and 4) and electrophoresed on a 5% acrylamide gel. The DNA of phage propagated on strain C600 is partially methylated at GATC sequences, whereas that from strain GM119 is not methylated, as seen in lanes 3 and 4, respectively.

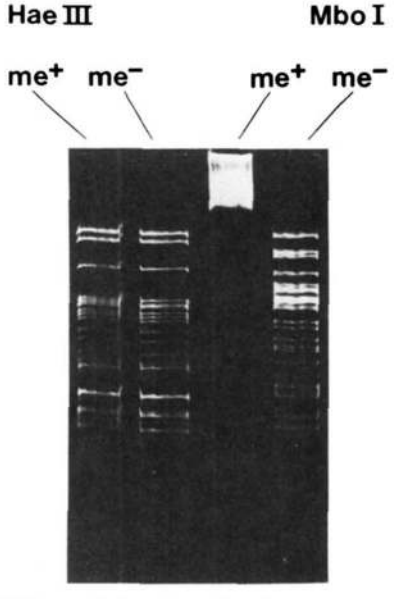

FIGURE 2.—*Restriction of highly methylated (me$^+$) and unmethylated (me$^-$) DNA of* λ*b2 bio69 c.* DNA was digested with *Hae*III (lanes 1 and 2) and *Mbo*I (lanes 3 and 4) and analyzed as in Figure 1. The DNA of phage propagated on strain C600 (*dam*$^+$) and additionally methylated *in vitro* with DNA adenine methylase is seen to be resistant to digestion with *Mbo*I (lane 3), indicating a high degree of methylation of GATC sequences.

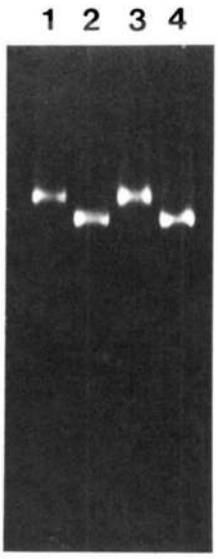

FIGURE 3.—Gel electrophoresis of separated chains. Separated DNA chains of λ*b2 bio69* (lanes 1 and 2) and λ*b2 bio69 c* (lanes 3 and 4). Separated DNA chains were applied in 0.1 M NaOH to a neutral 5% agarose gel. The DNA is from phage propagated on strain GM33 (*dam*-3). Heavy chains (lanes 1 and 3) migrate less rapidly than light chains (lanes 2 and 4). It is seen that the separated chains are intact and essentially free of contamination by complementary chains.

ylated chains in this series of transfections is the presence of 6-methylaminopurine at GATC sequences of the latter. The results of transfections in strains 152 (*dam*$^+$) and GM33 (*dam*) are shown in Table 3, parts A and B. As before, the presence or absence of the methylase in the host cells has no significant effect on the outcome. Upon comparison with the results for heteroduplexes constructed with partially methylated chains (Table 2), two striking differences are apparent.

First, the strand selectivity of mismatch repair is indeed enhanced. The fourth lines of Tables 2 and 3 show this most clearly. When partially methylated chains are used, about 25% of the plaques are *c*, the marker on the unmethylated chain (Table 2). In contrast, when highly methylated chains are used, only about 4% of the plaques are *c* and the percentage of + plaques rises from 73–74% to about 95% (Table 3). Thus, increasing the level of methylation on the methylated chain increases the strand selectivity of methyl-directed mismatch repair. By providing highly methylated chains in the heteroduplex, we more closely approach the situation at the growing fork of the bacterial chromosome, where GATC sequences on the template chains are fully methylated and those on the newly synthesized chains are transiently unmethylated.

The second change we note upon using highly methylated chains is the decrease in repair frequency of heteroduplex I when both chains are methylated. This result was unexpected, because in experiments using partially methylated chains and involving various mismatches methylation influenced only the strand selectivity of repair but not its total frequency at a given mismatch (M.

TABLE 3

Mismatch repair of heteroduplexes containing highly methylated chains

Heteroduplex			Plaques											
			A *dam*$^+$ host (152)				B *dam* host (GM33)				C *mutL* host (ES568)			
			c	Mixed	+	*n*	*c*	Mixed	+	*n*	*c*	Mixed	+	*n*
I		me$^+$/me$^+$	33%	32%	35%	210	37%	31%	32%	625	42%	38%	20%	314
c	*ℓ*	me$^-$/me$^-$	64	3	33	235	73	6	21	656	52	21	27	289
	h	me$^+$/me$^-$	95	3	2	190	96	2	2	543	66	18	16	229
+		me$^-$/me$^+$	4	2	94	203	3	2	95	834	22	28	50	404
II		me$^+$/me$^+$	29	26	45	215	35	33	32	532	34	35	30	204
+	*ℓ*	me$^-$/me$^-$	40	16	44	119	32	36	32	530	33	27	40	458
	h	me$^+$/me$^-$	38	29	32	226	33	40	37	897	34	37	29	337
c		me$^-$/me$^+$	33	30	37	223	28	38	34	835	30	30	40	276

RYKOWSKI and M. MESELSON, unpublished results). The percentage of mixed plaques from heteroduplex I with both chains fully methylated is close to the maximum value of about 35%, indicative of little or no repair (Table 3, line 1). It appears, therefore, that this repair system is nearly or completely inactive on fully methylated chains.

A control experiment was done to demonstrate that the resistance to repair attributed to full methylation was not due instead to some unsuspected effect of the methylation procedure or the subsequent strand separation and annealing of the enzyme-treated DNA. Aliquots of the me^+/me^+ partially methylated type I heteroduplex preparation were incubated in the complete methylation system, or with enzyme or *S*-adenosylmethionine omitted. Another aliquot received no treatment. The results, given in Table 4, confirm that it is the high degree of adenine methylation at GATC sequences, and not some other factor, which renders the heteroduplex resistant to repair.

Several genes of *E. coli*, including *uvr*D, *mut*H, *mut*L and *mut*S are thought to function in mismatch repair (NEVERS and SPAATZ 1975; RYDBERG 1978; GLICKMAN and RADMAN 1980). The repair frequency of heteroduplex I is sharply reduced in cells mutant for *uvr*D, although methyl-directed strand selection is still apparent (M. RYKOWSKI and M. MESELSON, unpublished results). We transfected the *mut*L strain ES568 with the same heteroduplex preparations used in the transfections of Table 3, parts A and B. As seen in part C, *mut*L has an effect like that of *uvr*D. The frequency of mixed plaques is increased, indicating a decrease in repair frequency, while methyl-directed strand selection is still present. No reduction in repair frequency is seen in ES4, the *mut*L^+ strain from which ES568 was derived (data not shown). If, in addition to the methyl-directed system, there were a different pathway able to repair the mismatch, there might exist mutations that would reduce repair frequency and also abolish methyl-directed strand selection. The fact that methyl direction is retained in *uvr*D and *mut*L is consistent with the possibility that there is no methyl-independent pathway for repair of this mismatch.

FURTHER DISCUSSION

We have shown that the methyl-directed strand specificity of mismatch repair previously studied in heteroduplexes with partially methylated chains is enhanced in heteroduplexes with chains highly methylated at GATC sequences by the enzyme DNA adenine methylase. This supports the hypothesis that repair works in a manner directed by the transient undermethylation of new DNA chains to eliminate errors in DNA synthesis (WAGNER and MESELSON 1976).

Further support for this hypothesis comes from studies of strains deficient in DNA adenine methylase and strains that overproduce it. If methylation fails to occur or if it occurs on new chains before repair can take place, strand selection should no longer be possible. These expectations are borne out by the observation of greatly elevated mutation rates in strains lacking DNA adenine methylase (MARINUS and MORRIS 1974; GLICKMAN, VAN DEN ELSEN and RADMAN 1978; BALE, D'ALARCAO and MARINUS 1979) and also in strains that overproduce

TABLE 4

Mismatch repair of heteroduplex I before and after methylation in vitro[a]

Source of DNA chains	Plaques			
	c	Mixed	+	*n*
C600*dam*$^+$	50%	3%	47%	443
Above heteroduplex preparation further methylated *in vitro*.	40	24	36	544
Above but minus:				
Enzyme	58	1	41	301
S-adenosylmethionine	57	3	40	449

[a] Heteroduplex I constructed from chains partially methylated *in vivo* in strain C600 (*dam*$^+$) was further methylated *in vitro* with DNA adenine methylase and used to transfect strain 152. Untreated samples of the same heteroduplex preparation and samples incubated in the reaction mixture minus enzyme or minus *S*-adenosylmethionine were also tested.

the enzyme (HERMAN and MODRICH 1981; M. G. MARINUS, personal communication).

A detailed model of the mechanism of methyl direction must await a biochemical analysis of the process and its components. It is clear, however, that strand orientation of the repair machinery must occur at a considerable distance along the DNA molecule from the site to be repaired. The average spacing of GATC sequences in λ and *E. coli* DNA is about 0.3 kb, and much longer spacings also occur in both. The extreme clustering of GATC sequences around the replication origin of *E. coli* and *Salmonella typhimurium* (MEIJER *ET AL.* 1979; SUGIMOTO *ET AL.* 1979; ZYSKIND and SMITH 1980) and the indication of preferential localization of these sequences at the ends of Okazaki fragments (GOMEZ-EICHELMANN and LARK 1977) may mean that the repair system is integrated into the replication complex itself. However, the repair frequency of a mismatch cannot be determined only by its distance from the methylation sites, since the two chemically distinct *c*/+ mismatches of heteroduplexes I and II are located at the same site but are repaired at very different frequencies.

It has recently been suggested that, regardless of whether mismatches are present, undermethylated strands may be susceptible to breakage and that such single-chain breaks direct a mismatch repair mechanism to operate preferentially on strands containing them (LACKS, DUNN and GREENBERG 1982). We consider this explanation inadequate to account for all of our observations. Our preparations of methylated and unmethylated DNA strands are equally intact (Figure 3 and data not shown). Moreover, the determination of strand specificity by breaks existing before transfection is ruled out by the effects we observe following hypermethylation *in vitro* using purified *dam* methylase (Tables 3 and 4). Even if the postulated breaks were to occur in unmethylated DNA after transfection, an untested supposition, such breaks could not be the sole determinant of mismatch repair. This follows from the observation that heteroduplex II, even with either or both strands unmethylated, is refractory to repair,

whereas its conjugate, heteroduplex I, differing only at the mismatch, is highly repairable. Thus, we have no reason to believe that, in *E. coli*, unmethylated DNA is preferentially susceptible to single-chain breaks or that such postulated breaks account for methyl-directed mismatch repair. Instead, our results and those of M. RYKOWSKI and M. MESELSON (unpublished results) are most simply explained by assuming that the *E. coli* repair system must sense both the mismatch and an unmethylated GATC site before it executes the chain breakage and excision characteristic of mismatch repair.

Mismatch repair has been extensively studied because of its possible role in gene conversion and the clustering of genetic exchanges known as localized negative interference (RADDING 1978). However, since highly methylated heteroduplexes can be resistant to repair, the state of methylation of recombining molecules should be carefully considered before conclusions are drawn concerning the role of this mismatch repair system in such genetic phenomena.

In most eukaryotes 5-methylcytosine, rather than 6-methylaminopurine, is present as the result of postsynthetic methylation of DNA. It is not known, however, whether this or any other means of distinguishing newly synthesized chains is used to direct a repair process for the elimination of replication errors in higher organisms analogous to the system we find in *E. coli*.

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