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**Citation:** Smith, Bradley T, Alan D Grossman, and Graham C Walker. "Visualization of Mismatch Repair in Bacterial Cells." *Molecular Cell* 8.6 (2001): 1197–1206. Copyright © 2001 Cell Press

**As Published:** [http://dx.doi.org/10.1016/S1097-2765\(01\)00402-6](http://dx.doi.org/10.1016/S1097-2765(01)00402-6)

**Publisher:** Elsevier

**Persistent URL:** <http://hdl.handle.net/1721.1/83855>

**Version:** Final published version: final published article, as it appeared in a journal, conference proceedings, or other formally published context

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# Visualization of Mismatch Repair in Bacterial Cells

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## Summary

We determined the localizations of mismatch repair proteins in living *Bacillus subtilis* cells. MutS-GFP colocalized with the chromosome in all cells and formed foci in a subset of cells. MutL-GFP formed foci in a subset of cells, and its localization was MutS dependent. The introduction of mismatches by growth in 2-aminopurine caused a replication-dependent increase in the number of cells with MutS and MutL foci. Approximately half of the MutS foci colocalized with DNA polymerase foci. We conclude that MutS is associated with the entire chromosome, poised to detect mismatches. After detection, it appears that mismatch repair foci assemble at mismatches as they emerge from the DNA polymerase and are then carried away from the replisome by continuing replication.

## Introduction

Maintenance of genomic integrity is crucial for survival and production of progeny. All organisms possess DNA repair systems that act to repair damage and avoid mutations. One such system is long-patch mismatch repair (MMR), which is conserved throughout nature and plays a vital role in genomic maintenance (Friedberg et al., 1995; Modrich and Lahue, 1996; Buermeyer et al., 1999; Eisen and Hanawalt, 1999). For example, inactivation of MMR genes in humans results in an increased susceptibility to colon and certain other types of cancer (Modrich and Lahue, 1996; Buermeyer et al., 1999). In addition to mutation avoidance, MMR in bacteria monitors homologous recombination during both transformation and conjugation and aborts those events that occur between diverged DNA sequences (Lacks et al., 1982; Rayssi-guier et al., 1989).

The primary role of MMR is to correct mismatched or unpaired bases (i.e., insertion-deletion loops) that escape the proofreading exonuclease of the replicative DNA polymerase (Friedberg et al., 1995). While replicative DNA polymerases have an extremely high degree of fidelity due to base selection and proofreading activities (Kunkel, 1992), they do make mistakes. The replicative DNA polymerase of *Escherichia coli*, DNA polymerase III holoenzyme (DNA pol III), has an intrinsic error rate of  $10^{-7}$  (Friedberg et al., 1995). *E. coli* has a 4.6 Mb genome; therefore, DNA pol III will, on average, incorporate an incorrect nucleotide every one to two replication cycles. To avoid introduction of mutations into the ge-

nome, these base-pair mismatches are repaired by the MMR system, and in *E. coli* MMR lowers the spontaneous mutation rate to  $10^{-10}$  (Friedberg et al., 1995).

In *E. coli*, MMR is initiated when a MutS homodimer recognizes a base-pair mismatch or an insertion-deletion loop (Su and Modrich, 1986; Parker and Marinus, 1992). After MutS associates with the DNA, it is then bound by a MutL dimer (Grilley et al., 1989), triggering the downstream repair events: discrimination of the newly synthesized DNA strand from the parental strand, excision of the newly synthesized strand (which contains the error), and rereplication of the gap generated by excision. MutS and MutL are conserved from bacteria to humans (Eisen and Hanawalt, 1999). In eukaryotes, the MMR recognition activities are carried out by two heterodimers of MutS homologs: MutS $\alpha$ , which consists of MSH2 and MSH6 and recognizes base-pair mismatches and small insertion-deletion loops (Drummond et al., 1995; Palombo et al., 1995), and MutS $\beta$ , which consists of MSH2 and MSH3 and recognizes larger insertion-deletion loops (Palombo et al., 1996; Genschel et al., 1998). MutL's role in eukaryotic MMR is primarily accomplished by a heterodimer consisting of two homologs: MLH1 and PMS1 (hMLH1 and hPMS2 in humans; Prolla et al., 1994).

In this study, we have used fusions of the green fluorescent protein (GFP) to MutS and MutL to investigate MMR from a cell biological perspective in the bacterium *Bacillus subtilis*. We have found that MutS is associated with the entire chromosome, poised to detect mismatched base pairs. Mismatches appear to be recognized by MutS as they emerge from the replisome. After detection, MutS and MutL form discrete foci that assemble at the DNA polymerase and are then carried away from the polymerase as it extrudes more replicated DNA. These foci likely represent the active sites of MMR because growth in the mismatch-inducing agent 2-aminopurine results in a replication-dependent increase in the number of cells with MMR foci.

## Results

### MutS-GFP Associates with the Chromosome and Also Localizes in Foci

We constructed a strain of *B. subtilis* expressing a MutS-GFP fusion protein (Table 3). The fusion gene was present in single copy at the *mutS* gene's normal position in the chromosome and was expressed from the native promoter. MutS-GFP was found, in vivo, to retain a substantial fraction of the wild-type protein's activity (see Experimental Procedures).

In exponentially growing cells, the fluorescence from MutS-GFP was present throughout most of the cell. Strikingly, in ~5% of cells, a focus of MutS-GFP was visible above this background fluorescence (Table 1 and Figure 1A). A strain lacking MutS-GFP exhibited a lower level of background fluorescence and did not have foci (data not shown). Because the nucleoid (the chromosome and associated proteins) normally fills much of

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Table 1. A Mismatch-Inducing Treatment Increases the Number of Cells with MutS-GFP and MutL-GFP Foci

Treatment <sup>a</sup>	Fusion <sup>b</sup>	Percentage of Cells with <i>n</i> Foci				
		0	1	2	3	4
None	MutS-GFP (649)	95	5	<1	–	–
	MutL-GFP (432)	81	19	<1	–	–
	Tau-GFP (207)	2	29	49	9	10
2-AP	MutS-GFP (588)	59	24	17	–	–
	MutL-GFP (719)	45	38	16	1	–
	Tau-GFP (275)	<1	34	54	7	5

<sup>a</sup>Cultures of BTS61 (MutS-GFP), BTS18 (MutL-GFP), and BTS8 (Tau-GFP) were grown and treated with 2-AP as described in Experimental Procedures.

<sup>b</sup>The total number of cells counted is indicated in parentheses.

the cell (Figure 2A), any MutS-GFP that is binding to the chromosome cannot normally be distinguished from protein that is free in the cytoplasm. To address this issue, we treated cells with the protein synthesis inhibitor chloramphenicol, which causes the nucleoid to condense (Zusman et al., 1973). One hour after addition of chloramphenicol, the majority of the MutS-GFP fluorescence was coincident with the condensed nucleoids (compare Figure 2B to 2A), indicating that most of the MutS-GFP in the cell is associated with the chromosome rather than free in the cytoplasm.

Our observations indicate that MutS has two modes of associating with DNA. In all cells, MutS appears to be distributed over the chromosome, a property that would be consistent with MutS constantly scanning the genome in search of mismatches. In some cells there is a second mode of binding that results in the formation of discrete foci. To further study the actions of MMR proteins in vivo, we examined the localization of MutL, which is also essential for MMR.

### MutL Localizes in Foci

A MutL-GFP-expressing strain was constructed similarly to the MutS-GFP strain (Table 3). MutL-GFP retains little or no activity in vivo (see Experimental Procedures). However, based on the observations described below, it appears that the fusion protein is still able to participate in protein-protein interactions relevant to its role in MMR. In contrast to the MutS-GFP strain, the strain expressing MutL-GFP did not exhibit a background fluorescence above that of a strain lacking a GFP fusion, and the background was unaffected by chloramphenicol treatment (compare Figure 2D to 2C). Nevertheless, like MutS-GFP, MutL-GFP did localize in discrete foci in a subset of cells (19%; Table 1 and Figure 1C). The MutL-GFP fusion was recessive with respect to MutL. A strain expressing both MutL and MutL-GFP did not exhibit an increased mutation frequency relative to wild-type and did exhibit an ~5-fold reduction in the number of cells with MutL-GFP foci relative to strains expressing only MutL-GFP (data not shown).

The difference between the percentage of cells with MutS-GFP (5%) or MutL-GFP (19%) foci could be due to the fact that the MutL-GFP fusion retains little or no MMR activity in vivo. In the absence of wild-type MutL, the inability to efficiently complete repair may cause the MMR complexes containing MutL-GFP to persist longer than those containing MutS-GFP, resulting in our observation that there were more cells with foci of MutL-GFP than MutS-GFP. Consistent with this, we found that in MutS-GFP-expressing cells that are *mutL* deficient (and therefore lack all MMR activity) the number of cells exhibiting MutS-GFP foci was 10% (924 cells counted), compared to 5% in cells that are *mutL* proficient (Table 1). Furthermore, many of the MutS-GFP foci in the *mutL*-deficient cells were brighter than the MutS-GFP foci seen in the cells expressing MutL (data not shown), suggesting that an inability to complete repair in the

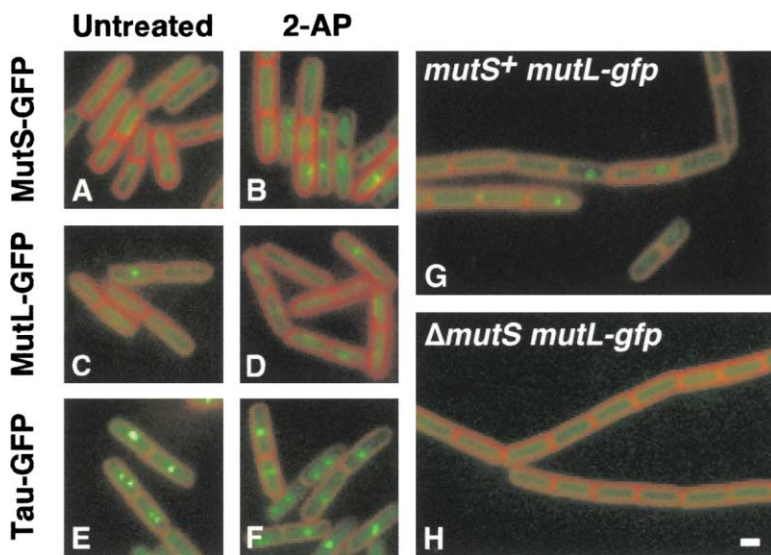


Figure 1. Localization of MutS, MutL, and DNA Polymerase in Untreated and 2-AP-Treated Cells

In untreated cells, MutS-GFP (A) fluorescence is present throughout the entire cell, and a subset of cells has a focus of MutS-GFP. Untreated MutL-GFP-expressing cells (C) do not exhibit background fluorescence above that of a cell lacking a GFP fusion, yet, like MutS-GFP, a focus of MutL-GFP is present in a subset of cells. In cells treated with 2-AP, the background fluorescence of MutS-GFP persists and the number of cells with foci increases (B). The number of cells with MutL-GFP foci also increases after 2-AP treatment (D). Tau-GFP foci (indicating the position of the replicative DNA polymerase) are present in all cells (E), and 2-AP treatment has no effect on their localization (F). Localization of MutL-GFP into foci was dependent on the presence in the cell of MutS, the mismatch-recognition protein (G). Cells lacking MutS did not exhibit MutL-GFP foci (H). Cultures of

BTS61 (MutS-GFP, [A and B]), BTS18 (MutL-GFP, [C and D]), and BTS8 (Tau-GFP, [E and F]) were grown and treated with 2-AP as described in Experimental Procedures. Cultures of BTS27 (*mutS<sup>+</sup> mutL-gfp* [G]) and BTS24 (*ΔmutS mutL-gfp* [H]) cells, which were not treated with 2-AP, were grown as described in Experimental Procedures. The fluorescence from the GFP fusion proteins is in green, and the cell membranes (FM-464 staining) are indicated in red. Scale bar: 1 μm.

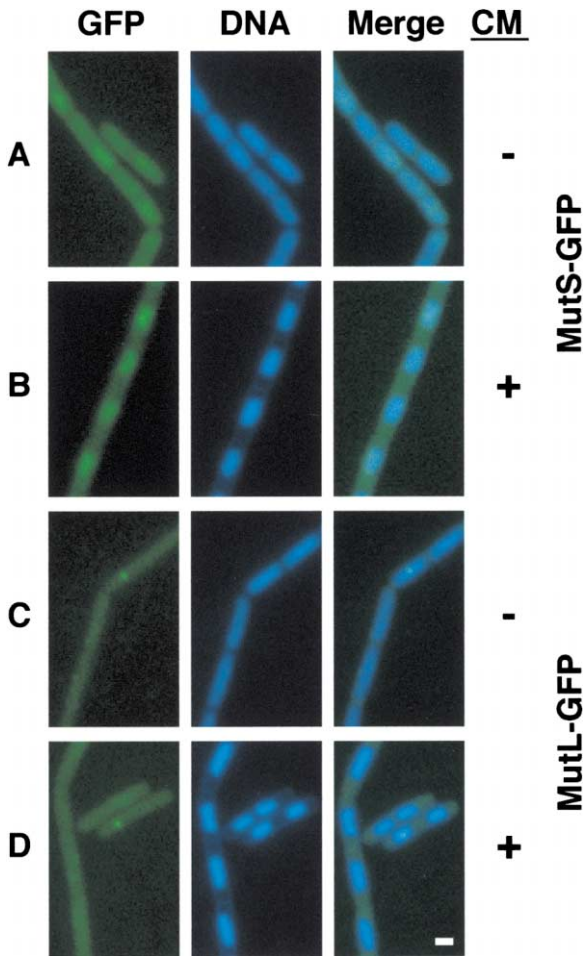


Figure 2. Visualization of MutS-GFP, MutL-GFP, and the Chromosome in Untreated and Chloramphenicol-Treated Cells

In living cells, the chromosome fills almost the entire cell volume, as does the background MutS-GFP fluorescence (A). Treatment of cells with the protein synthesis inhibitor chloramphenicol (CM), which condenses the nucleoids, resulted in a condensation of the majority of the background MutS-GFP fluorescence as well (B), indicating that most of the MutS-GFP is associated with the chromosome. A strain expressing MutL-GFP did not exhibit a background fluorescence above that of a strain lacking a GFP fusion and was unaffected by chloramphenicol treatment (compare Figure 2D to 2C). Cultures of BTS120 (MutS-GFP, [A and B]) or BTS18 (MutL-GFP, [C and D]) were grown and treated with chloramphenicol as described in Experimental Procedures. The fluorescence from the GFP fusion proteins is in green, and the chromosome is colored blue (DAPI staining). Scale bar: 1  $\mu$ m.

absence of MutL results in the accumulation of more MutS-GFP molecules at the site of the mismatch.

#### MutS Is Required for MutL-GFP Localization

Our observation that MutS-GFP foci formed in *mutL*-deficient cells indicates that MutL is not required for MutS focus formation *in vivo*. In contrast, we found that MutS was required for MutL localization. During exponential growth, *mutS*<sup>+</sup> *mutL-gfp* cells exhibited MutL-GFP foci (Figure 1G). However, in  $\Delta$ *mutS* *mutL-gfp* strains, the MutL-GFP foci were no longer observed (Figure 1H). Using immunoblotting, we determined that

the steady-state levels of MutL-GFP in both the *mutS*<sup>+</sup> and  $\Delta$ *mutS* strains were equivalent (data not shown). These findings are entirely consistent with biochemical studies of MMR, which have found that, after mismatch recognition by MutS, the MutS-DNA complex is bound by MutL.

#### MutS-GFP and MutL-GFP Form Foci in Response to Mismatches

To test the hypothesis that the MutS-GFP and MutL-GFP foci were the sites of active repair, we treated cultures with the mismatch-inducing agent 2-aminopurine (2-AP), an adenine base analog that is incorporated into nascent DNA by the DNA polymerase. 2-AP is able to form 2-AP·thymine base pairs and 2-AP·cytosine mispairs, the latter being substrates for MMR (Ronen, 1979; Glickman and Radman, 1980). After exponentially growing cultures of MutS-GFP- or MutL-GFP-expressing cells were treated with 2-AP, the percentage of cells in the population that exhibited one focus of MutS-GFP increased from 5% to 24% (Table 1 and Figure 1B), while the percentage that exhibited one MutL-GFP focus increased from 19% to 38% (Table 1 and Figure 1D). In addition, 2-AP treatment resulted in a dramatic increase in the number of cells with two foci, from less than 1% for both MutS-GFP and MutL-GFP to 17% and 16%, respectively (Table 1 and Figures 1B and 1D).

The steady-state levels of both the MutS-GFP and MutL-GFP fusion proteins, as assayed by immunoblotting, do not increase after 2-AP treatment (data not shown). This indicates that the appearance of more MutS- and MutL-GFP foci is not due to increased synthesis of the proteins but instead appears to be the result of the presence of more base-pair mismatches in the cell's DNA.

#### Inhibition of DNA Replication Decreases the Number of Cells with MutS-GFP and MutL-GFP Foci

The primary role of the MMR system is to correct errors generated by the replicative DNA polymerase. Therefore, we tested if the inhibition of DNA replication affected the localization of MutS- and MutL-GFP into foci. Since 2-AP is an adenine base analog, it must be incorporated into DNA by a polymerase to be mutagenic. We found that inhibition of replication by the replicative DNA polymerase-specific drug 6-(*p*-hydroxyphenylazo)-uracil (HPUra; Cozzarelli, 1977) prior to 2-AP treatment resulted in a decrease in the number of cells with MMR foci relative to parallel cultures that were treated with 2-AP but not HPUra. The number of cells exhibiting foci of MutS-GFP decreased from 46% (472 cells counted) to 18% (311 cells counted), and MutL-GFP foci decreased from 35% (353 cells counted) to 6% (319 cells counted). It is not known why the number of cells with MutS-GFP foci did not decrease as much as was seen with the MutL-GFP-expressing cells. Perhaps the rapid inhibition of the DNA polymerase and subsequent induction of the SOS response by HPUra (Iretton and Grossman, 1992) results in the formation of DNA structures that can be bound by MutS but that do not trigger the recruitment of MutL. It should be noted that DNA polymerase foci persist after HPUra treatment, albeit with slightly altered

Table 2. Turning Off Transcription of the *dnaA* Operon Reduces the Number of Cells with MutS-GFP and MutL-GFP Foci

	IPTG <sup>a</sup>	Percentage of Cells with Foci	
		Untreated <sup>b</sup>	2-AP Treated <sup>b</sup>
MutS-GFP	+	11 (428)	43 (355)
	-	4 (445)	3 (373)
MutL-GFP	+	16 (368)	30 (250)
	-	2 (381)	4 (256)

<sup>a</sup>BTS122 (*dnaA::P<sub>spac</sub>-dnaA<sup>+</sup> mutS-gfp*) or BTS34 (*dnaA::P<sub>spac</sub>-dnaA<sup>+</sup> mutL-gfp*) were grown +/- IPTG and +/- 2-AP as described in Experimental Procedures.

<sup>b</sup>The number in parentheses indicates the total number of cells (with and without foci) counted.

positioning and morphology (data not shown). This indicates that the reduction in MMR foci seen after HPUra treatment is not due to a wholesale disruption of cellular complexes involved in DNA metabolism but is specific to MutS- and MutL-GFP.

To confirm the above findings using another approach, we inhibited DNA replication by replacing the native promoter of the *dnaA* operon (which encodes both DnaA, the initiation factor, and  $\beta$ , the DNA pol III processivity factor) with an IPTG-inducible promoter. Removal of IPTG from the cultures results in an inhibition of DNA replication (Lemon and Grossman, 1998). We found that removal of IPTG caused a substantial reduction in the percentage of cells with MutS- and MutL-GFP foci, both in untreated cells (3- and 8-fold reductions, respectively; Table 2) and in cells treated with 2-AP after the removal of IPTG (13- and 8-fold reductions, respectively; Table 2). The fact that some MMR foci still persist in these two experiments after either HPUra addition or IPTG removal is likely due to residual replication perhaps carried out by repair DNA polymerases. Since MMR acts on errors generated by the DNA polymerase, the fact that fewer cells have foci of MutS- and MutL-GFP after the inhibition of DNA replication further supports the hypothesis that the MutS- and MutL-GFP foci that we observe are the sites of active mismatch repair and are not artifacts or inactive storage structures.

#### The Localization of MMR Foci with Respect to DNA Polymerase Foci

Since the above findings indicated that the MutS- and MutL-GFP foci represent sites of active repair and were dependent on DNA replication, we investigated the localization of the MMR proteins relative to that of the DNA replication factory (i.e., the replicative DNA polymerase). This factory has been found, in *B. subtilis*, to localize as a focus at mid-cell or as a pair of foci at 1/4 and 3/4 of cell length (Lemon and Grossman, 1998, 2000). We first determined the percentage of cells with MMR and DNA polymerase foci in untreated and 2-AP-treated cells. To localize the DNA polymerase, we used a Tau-GFP fusion. Tau dimerizes the two halves of the replicative DNA polymerase (Baker and Bell, 1998) and has been found to localize identically to that of the catalytic subunit of the polymerase, PolC (Lemon and Grossman, 1998, 2000). In contrast to MutS-GFP and MutL-GFP,

Tau-GFP foci are present in almost all cells regardless of treatment (Table 1 and Figure 1E). In addition, 2-AP treatment did not dramatically change the distribution of cells with one, two, or more Tau-GFP foci (Table 1 and Figure 1F).

Measurements of the positions of independently visualized MutS-, MutL-, and Tau-GFP foci indicated that the MMR foci did not always colocalize with the DNA polymerase foci. In untreated cells containing one focus of Tau-GFP, the focus was predominantly positioned at mid-cell (as has been reported previously in Lemon and Grossman, 2000; Figures 1E and 3A). The distributions of single MutS-GFP and MutL-GFP foci in untreated cells, on the other hand, were much broader than that of the polymerase and were positioned between the mid-cell and cell-quarter positions (compare Figures 3B and 3C to 3A). 2-AP treatment did not affect the appearance or positioning of Tau-GFP foci (compare Figure 3D to 3A and Figure 1E to 1F); single foci were still positioned at mid-cell. In 2-AP-treated cells, the broader distribution of single MutS-GFP and MutL-GFP foci relative to single Tau-GFP foci that was observed in untreated cells was again present (compare Figures 3E and 3F to 3D). In 2-AP-treated cells with two MutS- or MutL-GFP foci, those MMR foci are also more broadly distributed around the cell-quarter positions than are the twin Tau-GFP foci in similarly treated cells (compare Figures 3H and 3I to 3G).

To directly examine the localization of the MMR and DNA replication foci in the same cell, a strain expressing Tau-CFP and MutS-YFP was constructed (Table 3). We found that 48% of the MutS-YFP foci were touching or coincident with Tau-CFP foci (334 MutS-YFP foci were scored). MutS-YFP foci that did not colocalize with Tau-CFP foci were generally nearby them. Sample images of some MutS-YFP and Tau-CFP localization patterns are shown in Figure 4. In combination with the focus measurements described above, these data confirm that the MMR and DNA polymerase foci are not continuously colocalized.

## Discussion

### A Model for the Initial Steps of Mismatch Repair In Vivo

By using GFP fusions to visualize MMR proteins in living *B. subtilis* cells, we found that MutS, which recognizes base-pair mismatches, is associated with the chromosome in all cells yet also forms discrete foci in a subset of cells. The MutL protein forms foci in a subset of cells as well. Several pieces of evidence support the hypothesis that these foci represent accumulations of MMR proteins that have been recruited to the sites of mismatches and are actively involved in repair. First, the fact that MutL foci do not form in  $\Delta mutS$  cells is consistent with the results of biochemical studies which have shown that MutS accomplishes the first step in MMR, that of mismatch recognition (Su and Modrich, 1986; Parker and Marinus, 1992), and only after recognition has occurred does MutL become involved in repair (Grilley et al., 1989). Second, the number of cells with MutS and MutL foci increases after treatment with 2-AP, which increases the number of mispaired bases in the

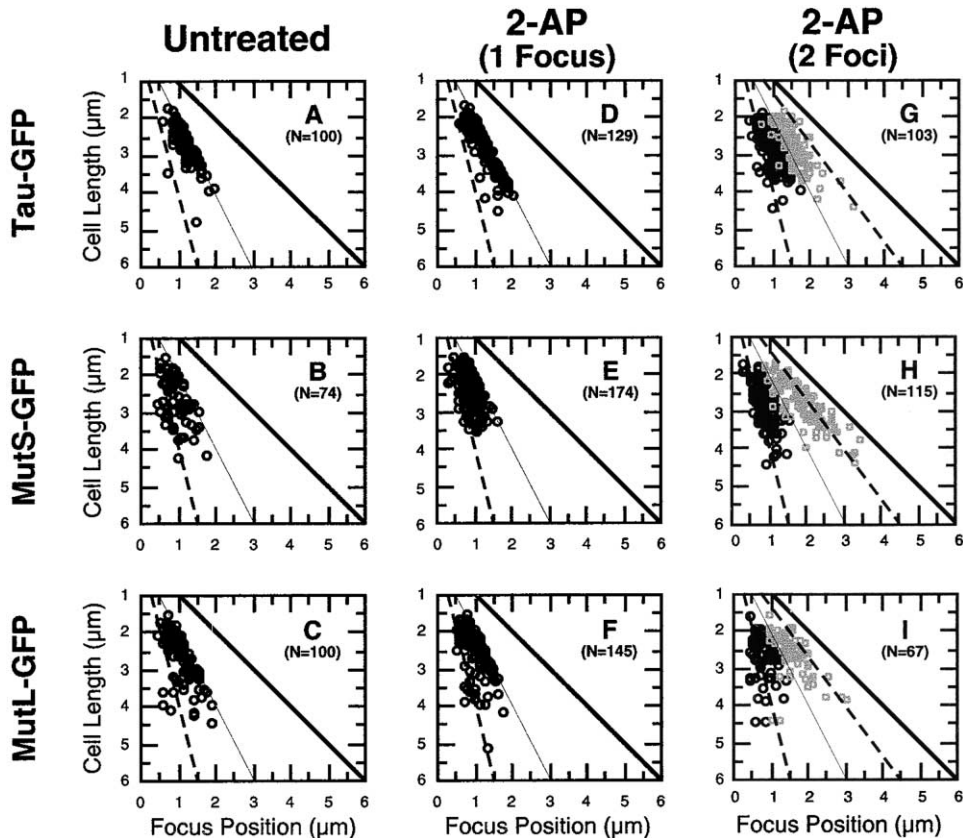


Figure 3. Positions of MutS, MutL, and DNA Polymerase Foci in Untreated and 2-AP-Treated Cells

In cells with one focus of either MutS-, MutL-, or Tau-GFP (representing the DNA polymerase), it was found that the MutS- (A) and MutL-GFP (B) foci were more broadly distributed between mid-cell and the cell-quarter position than the Tau-GFP foci (C), which were concentrated at mid-cell. The same was true in cells that had been treated with 2-AP (D–F). In 2-AP-treated cells with two foci of MutS-GFP (H) or MutL-GFP (I), the foci were positioned at roughly the cell-quarter positions and were again more broadly distributed than the foci in cells with two Tau-GFP foci (G). Cultures of strains BTS8 (Tau-GFP [A, D, and G]), BTS61 (MutS-GFP [B, E, and H]), and BTS18 (MutL-GFP [C, F, and I]) were grown and treated with 2-AP as described in Experimental Procedures. Foci were measured as described in Experimental Procedures. In (A)–(F), the black circles represent the positions in the cell of the single foci. In plots of twin foci positions (G)–(I), the black circle represents the position in the cell of the first focus, and the gray squares represent the position of the second. In all plots, the left-hand vertical axis marks the near cell pole, the dashed lines mark the cell-quarter positions, the thin line marks the mid-cell position, and the bold line marks the far cell pole. The number of foci measured is indicated in each plot.

genome. Furthermore, inhibition of DNA replication, which is the process by which base-pair mismatches are introduced into the genome, results in a large decrease in the number of cells with MutS and MutL foci. Our observation of MMR foci in cells that have not been treated with 2-AP is consistent with the fact that the DNA polymerase is continuously generating mismatches, albeit at a low frequency.

Unlike MutS-GFP, MutL-GFP retains little or no activity *in vivo*; therefore, it is formally possible that the MutL-GFP foci are artifactual accumulations of protein. However, we believe that the MutL-GFP foci do represent active sites of repair for the following reasons. First, the MutL-GFP foci are dependent on MutS. Second, their formation is inhibited by the expression of wild-type MutL. We interpret this second finding to mean that, in the absence of MutL, MutL-GFP is able to incorporate itself into repair complexes, but when MutL is present, the wild-type protein out-competes MutL-GFP for these complexes. Finally, the MutL-GFP foci are affected by 2-AP treatment and inhibition of DNA replication just as the MutS-GFP foci are.

The data presented here, combined with previous genetic and biochemical studies of MMR (Friedberg et al., 1995; Modrich and Lahue, 1996; Buermeyer et al., 1999), lead us to propose a model for the initial steps of MMR *in vivo*. MutS dimers are associated with the entire chromosome, poised to participate either in the correction of DNA replication errors or in the monitoring of homologous DNA recombination (another role of MMR), which is not linked with DNA replication. In support of this model, chromatin immunoprecipitation assays (which assayed only a small number of chromosomal sites) have found that the yeast MutS homolog, Msh2p, associates with yeast genomic DNA prior to any mutagenic or DNA-damaging treatments (Evans et al., 2000).

With respect to the correction of replication errors (which our studies have focused on), when the region of DNA with which the MutS dimer is associated encounters the replisome, the MutS dimer transiently associates with DNA pol III. Since a MutS- $\beta$  interaction has been observed using purified *E. coli* proteins (Lopez De Saro and O'Donnell, 2001), the association between MutS and the replisome is likely to involve the  $\beta$  pro-

Table 3. *B. subtilis* Strains Used

Strain	Genotype
PY79	prototroph SP $\beta^-$ (Youngman <i>et al.</i> , 1984)
JH642	trpC2 pheA1 (Perego <i>et al.</i> , 1988)
KPL213	JH642 <i>dnaA::P<sub>spac</sub>-dnaA</i> (cat) (Lemon and Grossman, 1998)
KPL382	JH642 <i>dnaX-gfpmut2</i> (spc) (Lemon and Grossman, 1998)
JCL270	JH642 <i>dnaX-cfp(w7)</i> (spc) (J. Lindow, unpublished data)
BTS8	PY79 <i>dnaX-gfpmut2</i> (spc)
BTS13	PY79 $\Delta$ mutSL::spc
BTS14	PY79 $\Delta$ mutL::spc
BTS18	PY79 mutL-gfpmut2 (spc)
BTS22	PY79 <i>amyE::P<sub>spac</sub>-mutL-gfpmut2</i> (cat)
BTS23	PY79 <i>amyE::P<sub>spac</sub>-mutL<sup>+</sup></i> (cat)
BTS24	PY79 $\Delta$ mutSL::spc <i>amyE::P<sub>spac</sub>-mutL-gfpmut2</i> (cat)
BTS25	PY79 $\Delta$ mutSL::spc <i>amyE::P<sub>spac</sub>-mutL<sup>+</sup></i> (cat)
BTS27	PY79 $\Delta$ mutL::spc <i>amyE::P<sub>spac</sub>-mutL-gfpmut2</i> (cat)
BTS28	PY79 $\Delta$ mutL::spc <i>amyE::P<sub>spac</sub>-mutL<sup>+</sup></i> (cat)
BTS34	PY79 <i>dnaA::P<sub>spac</sub>-dnaA<sup>+</sup></i> (cat) mutL-gfpmut2 (spc)
BTS56	PY79 mutS-gfpmut2 (spc) mutL <sup>-</sup>
BTS61	PY79 <i>amyE::P<sub>spac</sub>-mutL<sup>+</sup></i> (cat) mutS-gfpmut2 (spc) mutL <sup>-</sup>
BTS72	PY79 <i>amyE::P<sub>spac</sub>-mutL<sup>+</sup></i> (mIs)
BTS77	PY79 <i>amyE::P<sub>spac</sub>-mutL<sup>+</sup></i> (mIs) <i>dnaX-cfp(w7)</i> (cat)
BTS85	PY79 mutS-yfpmut2 (cat) mutL <sup>-</sup>
BTS107	PY79 <i>amyE::P<sub>spac</sub>-mutL<sup>+</sup></i> (mIs) <i>dnaX-crp(w7)</i> (tet)
BTS115	PY79 <i>amyE::P<sub>spac</sub>-mutL<sup>+</sup></i> (mIs) <i>dnaX-cfp(w7)</i> (tet) mutS-yfpmut2 (cat) mutL <sup>-</sup>
BTS120	PY79 <i>amyE::P<sub>spac</sub>-mutL<sup>+</sup></i> (mIs) mutS-gfpmut2 (spc) mutL <sup>-</sup>
BTS122	PY79 <i>dnaA::P<sub>spac</sub>-dnaA<sup>+</sup></i> (cat) mutS-gfpmut2 (spc) mutL <sup>-</sup>

cessivity factors that are associated with the DNA polymerase or that have accumulated on the lagging strand. This interaction may act to partly restrict the search window of MutS to DNA near the replisome, thereby contributing to its specificity for heteroduplex DNA and may also assist in strand discrimination (see below). We have not been able to directly compare the localizations of MutS and  $\beta$  because attempts to localize  $\beta$  in *B. subtilis* have yet to be successful (K. Lemon and A.D.G., unpublished data). After MutS recognizes the mismatch, the MutS-DNA complex is bound by MutL, and this tripartite complex is able to initiate the downstream steps of repair: strand discrimination, degradation of the newly synthesized DNA strand containing the error, and re-replication.

Because the MutS-MutL-DNA ternary complex is formed at or near the DNA replication factory (where mismatches are generated), it will initially colocalize with the replisome but will be carried away as the polymerase extrudes more DNA (Lemon and Grossman, 2000, 2001). The movement of the MMR complex away from the replisome is consistent both with the broader distributions within cells of MutS- and MutL-GFP foci with respect to Tau-GFP foci and with our finding that approximately half of MutS-YFP foci were touching or coincident with Tau-CFP foci when visualized in the same cell.

Based on our data, it is formally possible that the opposite situation is occurring: MMR foci could form at mismatches that have already been carried some distance from the replisome by ongoing DNA replication, and the MMR foci could then move back toward the DNA replication foci. However, we do not favor this model because it is not clear that the MMR complex is capable of actively translocating along the DNA. Of the three biochemical models for MMR (Allen *et al.*, 1997; Gradia

*et al.*, 1999; Schofield *et al.*, 2001), only one proposes that the MMR complex actively translocates along DNA (using the energy from ATP hydrolysis; Allen *et al.*, 1997), and the maximum rate observed was only 10%–20% that of DNA replication, which is occurring at  $\sim$ 1000 nucleotides per second (Baker and Bell, 1998). Rapid photo-bleaching of MutS-YFP has prevented us from carrying out time-lapse studies to directly confirm that the MutS-YFP foci form at the DNA polymerase foci and are then carried away.

Using indirect immunofluorescence in fixed cells, it has recently been found that two human homologs of MutS (hMSH3 and hMSH6) colocalize with PCNA (the eukaryotic DNA polymerase processivity factor) at DNA replication foci in the nuclei of human cells (Kleczkowska *et al.*, 2001). In contrast to our observations, this study found that the MMR proteins always appear to be coincident with the replication foci. This may reflect a different MMR mechanism in eukaryotic cells. Alternatively, the larger size and more complex nature of eukaryotic DNA replication foci (Cook, 1999; Leonhardt *et al.*, 2000) relative to the DNA pol III foci in *B. subtilis* (Lemon and Grossman, 1998) may mask any movement of the eukaryotic MMR complex away from the replisome.

#### Multiple MMR Complexes Assemble at a Mismatch

The fact that we observed visible foci of MutS- and MutL-GFP indicates that there is likely more than just one dimer of MutS- or MutL-GFP at the site of the mismatch. Unfortunately, the number of GFP fusion proteins needed to see a focus *in vivo* is not known and may be different for different fusion proteins. An accumulation of MMR proteins on the DNA flanking a mismatch is consistent with two of the three current biochemical models of MMR (Allen *et al.*, 1997; Gradia *et al.*, 1999). In these two models, after a dimer of MutS recognizes

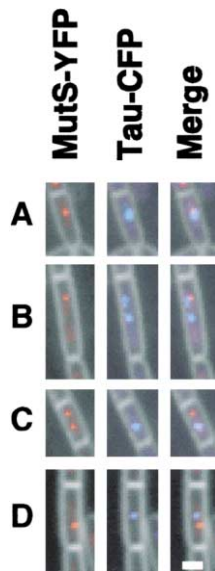


Figure 4. Simultaneous Visualization of MutS and DNA Polymerase Foci in the Same Cell

When visualized in the same cells, only 50% of the MutS-YFP foci were found to be touching or coincident with Tau-CFP foci (representing the localization of the replisome). (A–D) Samples images of BTS115 cells expressing both MutS-YFP and Tau-CFP. (A) A cell with one MutS focus that is coincident with a polymerase focus. (B) A cell with one MutS focus that is touching one of the two polymerase foci. (C) A cell in which only one of the two MutS foci is coincident with the single polymerase focus. (D) A cell in which the single MutS focus is not coincident with or touching the single polymerase focus. BTS115 was grown and treated with 2-AP as described in Experimental Procedures. The fluorescence from the MutS-YFP fusion proteins is in orange, the fluorescence from Tau-CFP is in cyan, and the cell membranes are colored gray (FM-464 staining). Scale bar: 1  $\mu\text{m}$ .

and binds to a mismatch, it moves away from the mismatch while remaining associated with the DNA. Release of the mismatch and movement away from it by MutS would allow subsequent recognition of the mismatch by additional MutS dimers, thereby allowing the visualization of MMR foci. These two models differ as to whether the movement of MutS along the DNA is due to active translocation (Allen et al., 1997) or passive sliding (Gradia et al., 1999). In the third biochemical model of MMR, MutS is capable of moving away from the mismatch, but it was proposed that this movement is normally inhibited by the action of MutL (Schofield et al., 2001). Since we propose that MMR focus formation is dependent on the movement of MutS away from the mismatch along the DNA, and since we found that MutS foci formed in the presence of MutL, our results are not consistent with this third model.

Since MutS-GFP foci formed in *mutL*-deficient cells, our results indicate that, *in vivo*, neither mismatch recognition by MutS nor the subsequent migration of MutS away from the mismatch requires MutL. We also observed MutL-GFP foci, but only in *mutS*<sup>+</sup> cells. Therefore, the MutL foci are likely to result from the association of MutL with the accumulated MutS dimers. These findings imply that, *in vivo*, MutL does not normally interact with MutS and only does so after MutS has recognized a mismatch.

### The Roles of the Replicative DNA Polymerase in MMR

The close association of the MMR machinery with the replisome that we observed, which had been suggested many years ago (Wagner and Meselson, 1976), may play an important role in strand discrimination. Since there is no damage *per se* at the site of a mismatched base pair, the MMR system must be able to discriminate the parental DNA strand from the newly synthesized one, which contains the incorrect base. *E. coli* targets repair to the newly synthesized strand by using the methylation status of GATC sequences as the signal to discriminate the newly synthesized DNA strand, which is transiently unmethylated, from the parental strand (Pukkila et al., 1983; Bruni et al., 1988; Claverys and Mejean, 1988). Methyl-directed strand discrimination requires the action of the Dam methylase and the MutH endonuclease (Friedberg et al., 1995). In contrast to the widespread presence of MutS and MutL in nature, the Dam and MutH proteins are found only in *E. coli* and a relatively small number of other bacteria (Eisen and Hanawalt, 1999). Therefore, most prokaryotes (including *B. subtilis*) and all eukaryotes must carry out strand discrimination by some other means.

Early studies of the *Streptococcus pneumoniae* MMR system, which functions in the absence of methylation, have suggested that strand discrimination may be accomplished by detecting the ends of the newly synthesized DNA strand at the replication fork (Lacks et al., 1982). Supporting this, prokaryotic and eukaryotic MMR proteins have been found to interact with DNA polymerase processivity factors ( $\beta$  in prokaryotes and PCNA in eukaryotes; Umar et al., 1996; Gu et al., 1998; Kleczkowska et al., 2001; Lopez De Saro and O'Donnell, 2001). Additionally, in eukaryotic systems PCNA has been shown to be involved in MMR prior to its expected role in post-repair DNA rereplication (Umar et al., 1996; Gu et al., 1998; Kleczkowska et al., 2001). Our finding that MMR complexes appear to assemble at DNA replication factories supports the model that strand discrimination occurs via interactions between MMR proteins and the replisome.

In addition to strand discrimination, the replicative DNA polymerase (DNA pol III) has also been implicated in rereplication, the final step of MMR. *In vitro* studies have found that extracts from *E. coli* strains carrying a temperature-sensitive allele of *dnaX*, which encodes both the  $\tau$  and  $\gamma$  subunits of DNA pol III, are defective for MMR at the nonpermissive temperature and can be complemented by the addition of purified DNA pol III holoenzyme (Lahue et al., 1989). Since the MMR foci that we observe likely represent active sites of repair, the fact that they do not continuously colocalize with DNA replication factories (as visualized by Tau-CFP foci) suggests that once the initial steps of MMR are accomplished (i.e., mismatch recognition and strand discrimination), the MMR machinery does not need to be associated with a replication factory to complete repair. However, it is formally possible that the MMR foci that are not coincident with the DNA replication foci have already completed repair and have yet to disassemble.

If DNA pol III is involved in rereplication *in vivo*, then our observation of MMR foci separated from DNA polymerase factories suggests that a small number of DNA



pol III complexes may dissociate from the replication factory to carry out rereplication. These polymerase complexes may not form visible foci because there are not enough complexes to visualize a focus or they do not remain assembled long enough. The dissociation of the polymerase holoenzymes from the replication factory may be mediated by interactions between the MMR proteins and DNA pol III. Due to the fact that the DNA replication factories are thought to contain more holoenzyme complexes than are required for chromosomal duplication (Lemon and Grossman, 1998), the holoenzyme that carries out rereplication need not have been engaged in chromosomal duplication. Since *B. subtilis* may have five or more DNA polymerases (Kunst et al., 1997), it is also possible that, in vivo, additional DNA polymerases can carry out rereplication in lieu of DNA pol III.

Our observations of the *B. subtilis* MutS and MutL proteins fused to GFP have offered new insights into the behavior of these MMR proteins in living cells. Efforts to elucidate the mechanism underlying MMR are ongoing, and what is learned in prokaryotic systems will undoubtedly be applicable to eukaryotic MMR and vice versa. In particular, the study of strand discrimination in bacteria that lack methyl-direction, such as *B. subtilis* and *S. pneumoniae*, will likely provide important insights into this crucial step of mismatch repair.

## Experimental Procedures

### Growth Media and Antibiotics

For microscopy, all strains were grown at 30°C in S7 defined minimal medium supplemented with 1% glucose and 0.1% glutamate (Vasantha and Freese, 1980; Jaacks et al., 1989) and with tryptophan (40 µg/ml) and phenylalanine (40 µg/ml). Mutation frequency assays were performed at 30°C using Luria-Bertani (LB) medium (Harwood and Cutting, 1992). Where needed, the following antibiotics were used: chloramphenicol (*cat*), 5 µg/ml; spectinomycin (*spc*), 100 µg/ml; tetracycline (*tet*), 12.5 µg/ml; erythromycin, 0.5 µg/ml and lincosmycin, 12.5 µg/ml, together (*mls*); and rifampicin, 100 µg/ml. 2-AP (Sigma) was used at 600 µg/ml. HPUra was used at 50 µg/ml and was a generous gift from G. Wright (University of Massachusetts Medical Center, Worcester, MA). Where needed, IPTG was used at 500 µM for *amyE::P<sub>spac</sub>-mutL<sup>+</sup>* and *-mutL-gfp* and at 50 µM for *dnaA::P<sub>spac</sub>-dnaA<sup>+</sup>*.

### Construction of *B. subtilis* Strains Expressing MutS-GFP and MutL-GFP

We constructed strains of *B. subtilis* that express translational fusions of GFP to the carboxyl termini of MutS and MutL. In each case, the fusion gene is the only version of the gene present in the cell, is located at the gene's natural position in the chromosome, and is expressed from its native promoter. All strains used in these studies are listed in Table 3 and were derivatives of PY79 (Youngman et al., 1984). All plasmids were transformed into PY79 using standard techniques (Harwood and Cutting, 1992). The *mutS-gfp* and *mutL-gfp* fusions were constructed using pKL147 as described (Lemon and Grossman, 1998) to generate BTS56 and BTS18, respectively. The *mutS-yfp* fusion was constructed similarly using pKL184 (K. Lemon and A.D.G., unpublished data) to generate BTS85. Deletions of the *mutSL* operon and of the *mutL* gene (leaving the upstream *mutS* intact) were constructed using pJL74 (LeDeaux and Grossman, 1995) to generate BTS13 and BTS14.

An *amyE::P<sub>spac</sub>-mutL<sup>+</sup>* construct was made by amplifying *mutL<sup>+</sup>* from PY79 and cloning it into pDR66 (Ireton et al., 1993) downstream of the IPTG-inducible *P<sub>spac</sub>* promoter. This plasmid was used to generate BTS23. BTS72, a *cat<sup>S</sup> mls<sup>R</sup>* derivative of BTS23, and BTS107, a *cat<sup>S</sup> tet<sup>R</sup>* derivative of BTS77, were constructed by transformation

with pCm::Er or pCm::Tet, respectively (Steinmetz and Richter, 1994).

To carry out the experiments in which we determined the localization of MutL-GFP in the presence and absence of MutS, we combined an *amyE::P<sub>spac</sub>-mutL-gfp* construct with *ΔmutL::spc* and *ΔmutSL::spc* to generate BTS27 and BTS24, respectively. The *amyE::P<sub>spac</sub>-mutL-gfp (cat)* construct was made as described above for the *mutL<sup>+</sup>* construct (*mutL-gfp* was amplified from BTS18) and was used to generate BTS22.

All subsequent strains (listed in Table 3) were constructed in PY79 by transformation of chromosomal DNA from the above strains and from KPL382 [*dnaX-gfpmut2 (spc)*] (Lemon and Grossman, 1998), KPL213 [*dnaA::P<sub>spac</sub>-dnaA (cat)*] (Lemon and Grossman, 1998), and JCL270 [*dnaX-cfp(w7) (cat)*] (J. Lindow, personal communication).

### Spontaneous Mutation Frequency Assays

To determine the in vivo activity of the MutS-GFP and MutL-GFP fusion proteins, the spontaneous mutation frequency of each strain was calculated by determining the number of rifampicin-resistant cells per colony-forming unit essentially as described (Haber and Walker, 1991). The average mutation frequency of each strain was determined from three to six independent cultures.

In *B. subtilis*, *mutL* is immediately downstream of *mutS* (Ginetti et al., 1996). Therefore, construction of *mutS-gfp* (BTS56) resulted in the inactivation of *mutL*. The spontaneous mutation frequency of BTS56 is ~300-fold higher than that of PY79 and is equivalent to that of BTS14. The *amyE::P<sub>spac</sub>-mutL<sup>+</sup>* construct was made to complement the inactivated *mutL* gene. The activity of this construct was tested in strain BTS28. It was found that BTS28 exhibited a 4.2-fold-higher spontaneous mutation frequency relative to PY79, which indicated that the *amyE::P<sub>spac</sub>-mutL<sup>+</sup>* construct was not quite as efficient in repair as the wild-type *mutL* gene. The *amyE::P<sub>spac</sub>-mutL<sup>+</sup>* construct was paired with *mutS-gfp* in all experiments unless noted.

To determine the in vivo activity of MutS-GFP relative to MutS, the spontaneous mutation frequency of BTS61 was compared to that of isogenic *mutS<sup>+</sup>* (BTS28) and *ΔmutS* (BTS25) strains. Relative to BTS28, BTS61 exhibited a 2.5-fold-higher mutation frequency, whereas BTS25 had a 66-fold-higher frequency. This indicated that MutS-GFP retained a substantial fraction of the activity of MutS. BTS18 (*mutL-gfp*) was compared to isogenic *mutL<sup>+</sup>* (PY79) and *ΔmutL* (BTS14) strains. Relative to PY79, BTS18 exhibited a 150-fold-higher spontaneous mutation frequency, whereas BTS14 had a ~300-fold-higher frequency. This indicated that MutL-GFP retained little or no activity. Neither alteration of the linker between the MutL and GFP moieties nor the fusion of GFP to the amino terminus of MutL increased the activity of the fusion protein.

### Cell Treatments and Scoring of MutS-GFP and MutL-GFP Foci

The effects of 2-AP or chloramphenicol on MutS-GFP and MutL-GFP were determined in an exponentially growing culture of cells expressing either MutS-GFP (BTS61 or the *cat<sup>R</sup>* derivative BTS120) or MutL-GFP (BTS18). The culture was split equally, and each half was treated with 2-AP or chloramphenicol, depending on the experiment. The parallel (untreated and treated) cultures were then allowed to grow for 1 hr, at which time aliquots were removed for microscopy. The visualization of MutS-YFP and Tau-CFP in the same cell was performed in 2-AP-treated BTS115 cells.

Experiments in which DNA replication was inhibited with HPUra were performed as follows. Cultures of BTS61 or BTS18 in mid-exponential growth were split, and one was treated with 50 µg/ml HPUra, which has been shown to block DNA replication (Brown, 1971). Fifteen minutes later, 2-AP was added to both parallel (+/-HPUra) cultures. One hour after 2-AP addition, aliquots from both parallel cultures were filtered (to remove HPUra which fluoresces in the GFP channel), and then viewed under the microscope.

Experiments in which DNA replication was inhibited by removing inducer (IPTG) from cultures of *dnaA::P<sub>spac</sub>-dnaA* strains were carried out essentially as described (Lemon and Grossman, 1998). 2-AP was added to the + and - IPTG cultures two doublings after removal of IPTG, and the cells were viewed two doublings after 2-AP addition. Since the *amyE::P<sub>spac</sub>-mutL<sup>+</sup>* construct uses the same IPTG inducible promoter as *dnaA::P<sub>spac</sub>-dnaA<sup>+</sup>*, the removal of IPTG from the culture

would have inactivated both the *mutL*<sup>+</sup> and *dnaA*<sup>+</sup> constructs. To avoid this complicating variable, BTS122 does not contain *amyE*::P<sub>spac</sub>-*mutL*<sup>+</sup>. The fact that *mutL* is inactivated in BTS122 does not markedly affect the overall result because we found that MutL is not required for MutS-GFP focus formation (see Results).

Foci were scored after colorization and merging of microscopic images (see below). Single foci were measured from mid-focus to the nearest cell pole, and in cells with two foci, both foci were measured to the same pole. All data presented here are cumulative from at least two independent experiments, each of which gave similar results.

#### Live Cell Microscopy

Microscopy of live cells was performed essentially as described (Lemon and Grossman, 2000). Aliquots of cells were stained with the vital membrane dye FM4-64 (Molecular Probes) and the DNA stain 4',6-diamidino-2-phenylindole (DAPI). The following Chroma filter sets were used: 41001 for GFP, 41029 for YFP, 31044v2 for CFP, 41002C for FM4-64, and 31000 for DAPI. Exposure times for GFP fusion proteins were as follows: MutL-GFP, MutS-GFP, and MutS-YFP, 4 s; and Tau-GFP and Tau-CFP, 3 s. Images were acquired, colorized, and merged using OpenLab software (Improvision) and were then transferred to Photoshop (Adobe) and Canvas (Deneba) for figure assembly.

#### Acknowledgments

The authors would first like to thank K. Lemon and P. Levin for providing considerable technical and scientific assistance during the course of this work. We thank the reviewers for their excellent comments. We thank T. Baker, S. Bell, J. Lindow, M. Marinus, B. Pellock, and A. Wright for critical reading of the manuscript, and J. Lindow for providing strain JCL270 prior to publication. We thank G. Wright for providing HPUra. We thank R. Tsien for providing the W7 variant of *cfp*. In addition, we thank all the members of the Walker and Grossman laboratories for their advice and support. This work was supported by Public Health Service grants CA21615 to G.C.W. and GM41934 to A.D.G. B.T.S. was partially supported by an NIH pre-doctoral training grant.

Received September 6, 2001; revised October 18, 2001.

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