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The Roles of DNA Mismatch Repair and Recombination in Drug Resistance: A Dissertation

Melissa A. Calmann
University of Massachusetts Medical School

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The Roles of DNA Mismatch Repair and Recombination in Drug Resistance

A Dissertation Presented

By

Melissa A. Calmann

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

December 2004

Biochemistry and Molecular Pharmacology
THE ROLES OF DNA MISMATCH REPAIR AND RECOMBINATION IN DRUG RESISTANCE

A Dissertation Presented
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Melissa A. Calmann

Approved as to style and content by:

__________________________
Michael R. Volkert Ph.D., Chairman of Committee

__________________________
Anthony R. Poteete Ph.D., Member of Committee

__________________________
Nicholas R. Rhind Ph.D., Member of Committee

__________________________
William R. Kobertz Ph.D., Member of Committee

__________________________
Graham C. Walker Ph.D., Member of Committee

__________________________
Martin G. Marinus Ph.D., Thesis Advisor

__________________________
Anthony Carruthers Ph.D.,
Dean of the Graduate School of Biomedical Sciences

Department of Biochemistry and Molecular Pharmacology

December 2004
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ABSTRACT

Cells have evolved different pathways in order to tolerate damage produced by different cytotoxic agents. Each agent reacts differently with DNA causing formation of different types of adducts, each eliciting the SOS stress response to induce different cellular repair pathways. One such type of substrate generated by cytotoxic agents is the DNA double strand break (DSB). The main pathway to repair such damage in the cell is through a process of recombination. In this thesis, I specifically examined the anti-cancer therapeutic agent cisplatin, which forms single- and double-strand breaks in DNA, and methylating agents, which are proposed to also be capable of forming such breaks. Neither type of agent can directly form these breaks; however, they leave a signature type of damage lesion which is recognized by different repair processes.

The mismatch repair (MMR) status of a mammalian cell or an Escherichia coli dam mutant relates directly to the sensitivity of the cells to the agents mentioned above. As the dam gene product plays an important role in this pathway and in other processes in the cell, when mutated, dam cells are more sensitive to methylating agents and cisplatin...
than wildtype. A combination of *dam* and either *mutS* or *mutL* restores resistance to the same agents to wild type levels. Therefore, mismatch repair sensitizes *dam* bacteria to these agents. The rationale for this comes from examining the viability of *dam* mutants, as *dam* mutants are only viable because they are highly recombinogenic. The presence of MMR-induced nicks or gaps results in the formation of DSBs that require recombination to restore genomic integrity.

Mismatch repair proteins inhibit recombination between homeologous DNA. Homeologous recombination (recombination between non-identical, but similar, DNA sequences) is only possible when the MMR proteins, MutS and MutL, are absent. It is postulated that this is because MutS recognizes the homeologous DNA and subsequently slows down or aborts recombination completely. The double mutant, *dam mutS/L* shows wild type levels of sensitivity to cisplatin because mismatch repair is no longer recognizing the adducts and recombinational repair is allowed to continue. Human cells behave in an analogous fashion to the bacterial *dam* mutant, showing sensitivity to cisplatin and methylating agents. When an additional mutation in a mismatch repair gene is present, the cells become as resistant as wild type. Therefore, the *E. coli dam* mutant is a useful model system to study this mechanism of drug resistance.

DNA containing cisplatin adducts or lesions resulting from methylation are substrates for other types of repair processes such as nucleotide excision repair and base excision repair; however they have also been implicated as substrates for MMR and recombinational repair. The goal of the work in this thesis was two-fold. The first was
to identify the gene products and mechanism necessary for repair of cisplatin damage by recombination. The second was to examine the mechanism of cisplatin toxicity, and specifically how MMR proficiency aids in the cytotoxicity of this drug by preventing recombination.

Using the duplicated inactive lac operon recombination assay, we were able to determine the requirements for spontaneous and cisplatin-induced recombination, the RecBCD and RecFOR pathways. We were also able to further postulate that the cisplatin-induced signature damage recognized by recombination was the double strand break, likely formed from fork stalling and regression or a subsequent collapse during DNA synthesis, thus requiring these pathways for repair. This observation led to the experiments involving examination of the mechanism of cisplatin toxicity and where MMR could inhibit specific steps of recombination with DNA containing cisplatin lesions. Low levels of cisplatin lesions slowed the rate of RecA-mediated strand transfer in vitro, likely due to its ability to form a large bend in the DNA. MutS bound to cisplatin lesions in the DNA during heteroduplex formation in the RecA strand exchange step of recombination, inhibiting branch migration, and aborting the reaction. In order for MutS to inhibit recombination with cisplatin lesions, the results in the work in Chapter IV, show that binding to the lesion requires the C-terminus of MutS to be present, possibly due to a requirement for tetramerization of the protein, a domain contained in the C-terminus of MutS. This antirecombination function is different than the mutation avoidance function of MutS, as binding of mismatches requires only dimers. This differential sensitivity for cisplatin versus a mismatch was further exemplified in
Chapter V, the experiments with *dna* mutants, where the greatest difference in sensitivity was observed for a *dnaE* mutant (catalytic subunit of polIII), which was as sensitive to cisplatin as a *dam* mutant, but fairly resistant to treatment with MNNG. This is indicative of the potency of a cisplatin adduct to block polymerase progression, versus a mismatch which poses little problem to synthesis. Recombination is invoked to repair DSBs caused by the cisplatin lesions through the RecBCD and FOR pathways after fork regression or collapse. A main conclusion from these studies is that a cisplatin lesion is processed differently than a mismatch. The mechanism of how a cisplatin lesion is processed, forming the DSB which invokes recombinational repair is still unclear and continues to be investigated.
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Statement of Contribution

The work presented in Chapter II was a result between a long standing collaboration of the Marinus and Essigmann labs. My contribution to the work in that chapter was at its initial phases, where I originally experimented with different conditions to optimize the qualitative lac operon recombination system for the correct number of bacterial cells for plating as well as, proper drug concentration. I performed the first analysis of the majority of the mutant strains exposed to cisplatin presented in Table 2-2, as well as obtained all of the transplatin control data for the same set of mutant strains.

In Chapter IV, the homeologous conjugational crosses (Table 4-2) were performed by Anetta Nowosielska, a postdoctoral fellow in the Marinus Lab. I am solely responsible for execution of all of the other experiments presented in that chapter and the remaining chapters, unless otherwise noted in the acknowledgement section following the end of each chapter.
CHAPTER I

INTRODUCTION

Drug-induced DNA damage.

In order to conserve the genome by preventing the passage of mutations from generation to generation, cells have evolved many pathways of repair. Basic understanding of these pathways is largely due to studies involving the Escherichia coli bacterium. DNA can undergo damage and thus sustain mutation from exposure to exogenous or endogenous sources, several of which will be discussed in detail. Cells have evolved intricate and redundant pathways to repair damage inflicted by a variety of cytotoxic agents, including exposure to chemicals and radiation. Each type of agent or their metabolites reacts with DNA, leaving a variety of adducts or base modifications, which elicit a cellular response to signal molecules for repair (1). When these DNA modifications are not repaired, their presence can ultimately lead to double strand break (DSB) formation in DNA (see below). Persistence of DSBs in DNA from lack of repair can cause cell death. Agents that induce DSBs in DNA can be naturally occurring, such as nitric oxide damage or ionizing radiation, as well as some used in therapy of neoplastic diseases, as well as xenobiotics in the environment. The research in this thesis focuses on examples of the last two, therapeutics for neoplastic diseases and xenobiotics, so naturally occurring double strand break formation will not be discussed further.
Cisplatin (cis-diaminodichloroplatinum II) is an effective anti-tumor agent used to cure testicular, ovarian and other types of cancers. It is greater than 90% effective for the treatment/eradication of testicular tumors, though the mechanism of its clinical efficacy is not completely understood (3). The reaction between cisplatin and DNA produces intra- and inter-strand crosslinks (4), in a ratio of 10,000-20,000 adducts per molecule in the human genome (5) and about 80 adducts per *E.coli* genome using the average clinical dosage (D. Froim personal communication). The adducts predominantly formed are intrastrand crosslinks between adjacent guanines, 1,2-d(GpG) (65% of total adducts), between adjacent guanines and adenines 1,2-d(ApG) (25%), and between guanines separated by a base 1,3-d(GpNpG) (5-10%), where N is any base (6-8). Interstrand crosslinks comprise about 2% of the total adducts formed and a small amount of monoadducts are formed. Cisplatin lesions block the progress of replicative polymerases including the PolIII holoenzyme and Poll. Cisplatin lesions can be bypassed by other polymerases used for translesion synthesis, but these polymerases often pair a platinated base incorrectly, where for example, an adenine or thymine will often be incorporated across from a guanine that has been reacted with cisplatin. This type of lesion is called a compound cisplatin lesion, while the correctly incorporated base opposite a base involved in a cisplatin crosslink is called a simple lesion (9). Compound lesions are most predominantly formed with the 1,2- adducts upon replication (9), however both simple and compound lesions are still cytotoxic. A diagram of the intra- and interstrand adducts produced by the cisplatin-DNA reaction is shown in Figure 1-1A and a ribbon diagram
Figure 1-1. **Schematic representation of cisplatin-DNA reaction.**

A. (Top) Chemical structure of cisplatin.
(Bottom) Types of adducts formed by reacting cisplatin with DNA (10).

\[
\begin{align*}
\text{H}_3\text{N} & \quad \text{Pt} \quad \text{NH}_3 \\
\text{Cl} \quad \text{Cl} & \quad \text{Cisplatin}
\end{align*}
\]

B. Ribbon diagram of a cisplatin adduct in DNA.
There is a large bend in the DNA created by the presence of cisplatin.
DNA strands(orange), Pt (blue), G-G bases (red), NH\textsubscript{3} (yellow)

Gelasco and Lippard *Biochem* 1998(2)
representation of a cisplatin lesion in DNA in Figure 1-1B. The ribbon diagram shows that the presence of cisplatin in DNA distorts the double helix DNA structure, as the crosslinking between the bases with the centrally located platinum molecule between them creates a large 50 degree bend in the DNA, which de-stacks the crosslinked bases and significantly widens the minor groove (11-13). This kink in the DNA prevents DNA replication by blocking polymerases during synthesis (14). The trans isomer of cisplatin, transplatin, also produces a spectrum of N7-intrastrand and interstrand crosslinks in DNA (6, 15). Transplatin, however is not used for clinical treatment of cancer, as it does not seem to have any biological relevance in regards to toxicity needed for tumor treatment. Presumably this is due to its ability to form adducts, as transplatin is restricted from forming 1,2-intrastrand crosslinks by geometric constraints (16). Thus it is postulated that the ability of cisplatin, and therefore the inability of transplatin to form these adducts, to form the 1,2- intrastrand crosslinks are the biologically relevant adduct enabling cisplatin to be used as an effective therapeutic (17). Transplatin and cisplatin can both block polymerase progression, although the concentration required for transplatin is much greater; this is likely due to the inability of transplatin to form the 1,2-intrastrand crosslink. This suggests that cytotoxicity is not due entirely to inhibition DNA synthesis, and the biological response to the 1,2-intrastrand crosslink must directly relate to the cytotoxicity of cisplatin (4), but the mechanism of how it does so is still not yet elucidated. Regardless, experiments employing transplatin still serve as a useful control compound to study cell sensitivity to cisplatin.
Methylating agents are also widely used as chemical therapeutics for cancer. Laboratory derived agents such as N-methyl-N’-nitro-N-nitrosurea (MNU) or N-methyl-N’-nitro-N-nitrosoguanidine (MNNG) and their clinical counterparts, agents such as dacarbazine, procarbazine, streptozoticin, and temozolomide have been studied extensively to determine the mechanism of their effectiveness. The cytotoxicity of methylating agents is due to their ability to introduce specific lesions into DNA, which can be mutagenic or lethal. Methylating agents can react with DNA at twelve different sites on DNA bases, including all exocyclic oxygens and some ring nitrogens (18). Oxygen atoms in the sugar phosphate backbone can also be subject to methylation by these agents (18). Addition of methyl groups to bases can severely diminish their ability to base pair with other bases, leading to mismatches. Similar to cisplatin containing DNA, problems can arise once the replication fork encounters these lesions, leading to replication fork collapse, double strand break formation and cell death if the DNA is not repaired (Calmann, Nowosielska, and Marinus, unpublished work). Methylated bases, like O6-methylguanine, do not cause any helical distortion when they are incorporated into DNA (18). However, frequent mispairing with O6-methylguanine occurs during replication which can lead to a futile cycling of repair, (19-21) (see Figure 1-10) causing fork stalling and DSB formation. Several proteins repair such damage in DNA and will be presented next. However, residual lesions persist in DNA especially at high levels of methylation, which are believed to make these agents favorable for eradicating tumors.
Despite the high percentage of testicular cancers curable by cisplatin and courses of treatment including methylating agents, a large drawback of these drug regimens is the development of drug-resistant tumors. Characterization of these tumors commonly reveals mutations in genes encoding repair proteins. The importance of this will be discussed in a later section of this Introduction.

**Ensuring the genomic integrity of DNA.**

DNA mismatch repair (MMR) acts to preserve the fidelity of the genome and is conserved from bacterial species to humans. It does so by its ability to recognize and repair base mismatches and small segments of DNA containing insertions/deletions of bases which may give rise to frameshifts. Mismatched base pairs arise during replication, where the replicative polymerase, PolIII and accessory proteins comprising the holoenzyme, can misincorporate non-complementary bases while synthesizing the new DNA strand. The error frequency of the holoenzyme to incorporate non-complementary bases into DNA is approximately 1/100,000 bases synthesized (10^-5) (22). When misincorporated bases are replicated, purine-pyrimidine mispairs generate transition mutations, while purine-purine or pyrimidine-pyrimidine mispairings are responsible for transversion mutations. The MMR system can recognize and repair these types of mismatches with a differential specificity, as the G-T mismatch is recognized and repaired with greatest affinity, while the C-C transversion is poorly recognized by MMR and is repaired with lowest efficiency (23). Mismatches A-C, G-G, A-A, T-T, C-T, and G-A are repaired with an intermediate efficiency, in order from highest to lowest
specificity (23). MMR also recognizes small insertion or deletion mutations of 1-4 nucleotides which can result from slippage of one DNA strand along the other during replication(24-26). These small insertions/deletions are recognized by MMR with greater affinity than even the G-T mismatch (24). The MMR proteins can also recognize lesions produced by methylation or oxidative damage to DNA, such as O\textsuperscript{6}-methylguanine and 8-oxoguanine. These lesions usually mispair upon replication which causes initiation of MMR (21, 27). MMR proteins can also bind the 1,2-cisplatin lesion in DNA (9, 28). The importance of mismatch recognition of lesions produced by drug treatments will be presented in detail later in the Introduction.

**DNA mismatch repair model.**

Much of what is known about the mechanism of mismatch repair is derived from studies of *E. coli* as diagrammed in Figure 1-2 (29). At the top left of the figure, there is a segment of newly replicated DNA, containing a mismatch represented by the boxed M. Newly synthesized DNA is methylated only on the parental strand, indicated by the Me in the diagram, at the N6 position of each adenine, in the sequence GATC, by the product of the dam gene (30). The new daughter strand has yet to be Dam methylated, due to a short lag period between replication and the methylation step, which in turn, produces a hemimethylated DNA structure (30).

MutS, a 95 kD polypeptide, which exists in solution as a mixture of mostly dimers and tetramers, specifically recognizes and binds to the mismatched base (31). The affinity
Figure 1-2. The current model for DNA mismatch repair in *E. coli*.

In the figure below, a hemimethylated segment of DNA with a mismatch (boxed M) is recognized and bound by dimers of MutS protein. MutS recruits dimers of MutL protein and a monomer of MutH protein, creating a ternary complex on the DNA with a theoretical looping conformation. The latent endonuclease activity of the MutH protein is activated once in the complex, causing it to nick the DNA at the nearest unmethylated GATC site. Once nicked, UvrD helicase unwinds the DNA, allowing for directional-specific exonucleases to load onto the DNA to excise a patch of DNA which includes the mismatch and up to 1,000 additional bases. Note in this model excision is occurring 3’ to 5’ which requires use of Exo I/X. The large gap remaining is re-synthesized by the loading of the replicative polymerase, polIII holoenzyme, and is sealed by DNA ligase. At this time, Dam methylase can methylate the new strand of DNA at the unmethylated GATC site, as the mismatch has been repaired (figure adapted from (29)).

of the protein for the mismatch varies depending on the mismatched base as does the efficiency of correction (23). In this model MutS is represented as a dimeric species, but there is no evidence to presume that MutS definitively binds as a dimer to the mismatch. Recent studies and evidence presented in this work, postulate that MutS likely binds as a tetramer, but for historical purposes it will be represented here as a dimer (32, 33). Once MutS binds the mismatch, it recruits a 70 kD protein, MutL, as a dimer, to the site (34). The exact role of MutL has not been elucidated as yet, but it is thought that MutL acts as a bridge protein from MutS to other proteins in the pathway, including MutH, which is recruited to the complex after the MutS/L interaction is established (35). The MutL protein has no characteristic functional domains or activities except a low ATPase activity, which was not discovered until recently (36, 37). It is speculated that MutL in fact has several roles, including acting as a stabilizing factor allowing MutS to remain bound to the mismatch, and is likely the key communicator between MutS and other proteins involved in excision and re-synthesis later in the pathway (34, 37). MutL only binds specifically to DNA in the presence of MutS and it also directly interacts with the UvrD protein later in the pathway (38, 39). How MutL provides its postulated communicative role is unknown, but it is known that in its absence, the MMR pathway cannot function.

Once the ternary MutS/L/H complex is formed, the latent endonuclease activity of MutH is activated, allowing for MutH to produce a nick in the DNA at the nearest unmethylated GATC sequence of the hemimethylated DNA structure (35, 40, 41). MutL
enhances the endonucleolytic activity of MutH, for in its absence in vitro, the activity of the MutH protein in the presence of DNA is poor (42). The role of MutH in the initiation of repair is critical, as the nearest unmethylated GATC site can be one kilobase or more away from the site of the mismatch (29, 43, 44). In vitro, a precisely placed nick in the DNA can substitute for the MutH-induced nick allowing for repair in its absence (43). At the site of MutH incision, the UvrD protein is loaded onto the DNA (38). UvrD is a helicase which unwinds the DNA allowing exonucleases to access the DNA to degrade it. Degradation begins at the site of incision, the GATC site, and continues through the mismatch, including a large number of bases past the mismatch (40, 44, 45). As the GATC site can reside 5' or 3' to the mismatch, the UvrD helicase has the inherent ability to determine the directionality needed for repair, as incision at a GATC 5' to the mismatch requires RecJ or Exonuclease VII, while a nick 3' to the mismatch requires Exonuclease I or X (46). The availability of four different exonucleases preserves the fidelity of the process by exploiting the bi-directional capability of the system. In the absence of one or more exonucleases, a different exonuclease can substitute for it in the repair process as there is a redundant exonuclease for each direction (47).

After excision, a gap in the DNA remains and needs to be re-synthesized. In the in vitro reaction, this is accomplished by addition of the polIII holoenzyme complex, which is the polymerase used for replication. No other polymerase was capable of substituting for polIII in the in vitro MMR assay (29). PolIII holoenzyme will load onto the DNA, and with high fidelity, synthesize bases to fill the gap. The nick that remains
after re-synthesis is sealed by DNA ligase and the mismatch is repaired. Dam can load onto the DNA and methylate the new daughter strand and the fidelity of the genome has been preserved this round of replication. MMR in eukaryotic cells is presumed to be similar to the bacterial process as there are homologs of the major proteins, MutS and L, however, there is no homolog for MutH so the mechanism for strand nicking is still unknown (48, 49).

**The role of MMR in homeologous recombination.**

Homologous recombination is another process by which DNA damage can be repaired. In order to repair the damage, the cell uses an intact undamaged homologous duplex chromosome, whose strands can aid in fixing the damaged strand. The hallmark of the recombination process is the formation of a heteroduplex joint molecule which contains one DNA strand from each of the recombining partner DNAs (50). The process is diagrammed in Figure 1-3 with a DNA molecule which has formed a double strand break which then pairs with an intact homologous partner. The two molecules exchange at regions of sequence identity. Resolution of the junctions formed by the pairing of these molecules separates the chromosomes from one another and the resulting strands contain segments of DNA from each of the chromosomes. DNA sequence homology is an essential requirement for homologous recombinational repair, and in *E. coli*, RecA protein searches for regions of sequence identity between the DNAs for alignment to initiate the reaction (50). Once the intact chromosome is aligned with the chromosome needing repair, crossover between the molecules occurs, catalyzed by RecA, as well as
The presence of a cisplatin lesion in DNA will cause the replication fork to stall during synthesis. This can result in formation of a double strand break (red) which is repaired by homologous recombination using an intact, undamaged chromosome (blue).
other cellular proteins. The heteroduplex molecule containing strands from both recombining partners undergoes branch migration and resolution of the molecule will occur, thus repairing the DNA (1). The efficiency of recombination is controlled by the degree and length of sequence similarity. In vivo, the recombination reaction requires near perfect homology and as little as 10% sequence divergence can cause the reaction to cease. In recombination simulation reactions in vitro, E. coli RecA protein can tolerate up to 30% sequence divergence before the reaction is aborted (50).

Recombination also provides a means for transfer of genetic information between segments of DNA. As very little sequence divergence can be tolerated in the recombination process, mismatch repair is essential for fidelity, and any mismatches generated during the alignment step of recombination must be repaired. MMR has been implicated as the major process which prevents recombination events between similar but non-identical DNA sequences or species, in a process termed homeologous recombination (51-53). An example of homeologous recombination is the recombination reaction with the E. coli and Salmonella typhimurium species, which are 15% divergent in DNA sequence. MMR plays a significant role in limiting recombination between these two species (antirecombination). As alignment of the DNAs from these two species produces many mismatches, MutS protein binds to the mismatched bases to initiate the MMR process, thus blocking the progression of the recombination reaction (54). In strains where MMR is absent, conjugational or transductional crosses between E. coli donors and Salmonella recipients produce an increased number of recombinants in a recA
dependent manner (52). Mutations in the *mutS* or *mutL* genes in the recipient strains increase recombination frequency by 1,000-fold. Mutations in *mutH* increase interspecies recombination also, but only about 20-fold in comparison (52). The role of MMR in antirecombination is also evident in other species, where in *Bacillus* strains for example, a single mismatch with an otherwise identical sequence can inhibit transformation (55). Antirecombination has been documented in murine models as well as other eukaryotic organisms. *In vitro*, antirecombination has also been reconstituted using the RecA-catalyzed strand exchange reaction. This was first documented using the M13 and fd phages which are 3% divergent in sequence (56). The basic three strand reaction is outlined in Figure 1-4 (56). In brief, in the presence of ATP and RecA protein, a single stranded circular DNA molecule and a linear duplex DNA molecule can form a heteroduplex molecule containing the circular DNA and the homologous single strand from the linear duplex. The end product of the reaction is a nicked circular duplex. The rate of this reaction is much slower than a reaction which uses completely homologous substrates, however, over time, the product yield is nearly identical. Upon addition of MutS to the M13 x fd reaction, the rate and yield of heteroduplex formation, and thus nicked circular product, is reduced significantly (56). When MutL, in the presence of MutS, is added to the same M13 x fd strand transfer reaction, product formation is almost completely abolished (56). The steps at which mismatch repair inhibits strand exchange are at the initiation of heteroduplex formation by RecA, and branch migration by RuvAB (54, 56), where the Mut proteins prevent displacement of the linear single strand product and is evident by an accumulation of intermediate structures
Figure 1-4. *In vitro strand exchange reaction* (56).

Linear duplex M13 phage DNA and single stranded circular fd DNA in the presence of ATP, SSB and RecA proteins, will form heteroduplex molecules in the form of branched intermediates, which will be resolved into an open/nicked circular product with the remaining non-homologous single strand.

**RecA-catalyzed M13-fd DNA exchange**

```
5' M13 + fd
3' linear dsDNA

RecA | SSB, ATP

branched intermediates

5' open circular heteroduplex + 5' linear ssDNA
```
which can be visualized by agarose electrophoresis (56). It is important to note that neither MutS nor MutL addition prevent the same reaction with completely homologous substrates (56),(52). How MutS/L inhibit branch migration during in vitro strand exchange, as well as during interspecies recombination, is still not understood.

**Mismatch repair, human tumors and drug resistance.**

DNA repair processes form an important line of defense against cancer by preventing mutations which may induce tumorigenesis. Therefore, it is not surprising that people with mutations in genes for repair, are more susceptible to cancer. Werner and Bloom’s Syndrome, Xeroderma Pigmentosa, and A-T (Ataxia-Telangiectasia) are all diseases associated with mutations affecting various repair functions which can allow for predisposition to cancers (57). Inactivation of MMR leads to a strong mutator phenotype in *E. coli* cells (58), and in humans predisposes cells to the development of tumors. In humans the high frequency of mutation is mapped most often to short repetitive DNA sequences known as microsatellites. These mutations lead to microsatellite instability because these sequences are prone to polymerase slippage during replication, thus causing mutations that result in cancer development (59-65). HNPCC (Hereditary Nonpolyposis Colon Cancer) is a disease resulting from microsatellite instability of dinucleotide repeats caused by mutation in the *hMSH* or *hMSL* genes, the human homologs of the bacterial MMR genes, *mutS* and *mutL* (66, 67). Humans and other eukaryotes have multiple MMR genes encoding several proteins, as opposed to *E. coli* which only has MutS and MutL, so mutation in any number of the human mismatch
repair genes can lead to cancer formation (68). Characterization of the tumors of individuals with mutated MMR gene(s) show genes with a high frequency of insertions and deletions, which would normally be able to be repaired by MMR. This is directly related to the high spontaneous mutation rate seen in hMSH or hMLH mutants from uncorrected errors and parallels the MMR- deficient phenotype characteristic of the E. coli system.

In addition to the high susceptibility to develop cancer from mutations in mismatch repair genes, deficiency in MMR also contributes to the formation of tumors resistant to treatment with cisplatin and clinical methylating agents such as dacarbazine and temozolomide (62). The correlation between mismatch repair deficiency and methylating agent and cisplatin resistance was first documented in experiments using E. coli as a model system to study the effects of these drugs. E. coli dam mutants are sensitive to the cytotoxic effects of laboratory derived methylating agents and cisplatin (21, 69). However, if a mismatch repair deficiency is present in addition to dam, the drug sensitivity reverts to resistance, at levels equivalent to that of a wildtype cell (21, 69). As dam directs the mismatch repair process to repair a mismatch in the new daughter strand of DNA, in its absence MMR incision by MutH can occur on either the newly synthesized strand or the parental template. When MMR is non functional because of mutations is the mutS or mutL genes, mutations, or in this case drug lesions, persist in the DNA and are replicated, which can lead to further mutation.
For human cells, the drug sensitivity to cisplatin and MNNG is similar to that of an *E. coli dam* mutant. Human cells are sensitive to both treatments, which enables these drugs to be used clinically in treating tumors (3, 70). However, a subset of cisplatin and methylating agent-resistant tumors that develop may be the result of mutations in the human homologs of the mismatch repair genes (60, 62, 65, 66, 71, 72). Of importance is that in studies with human carcinoma cell lines, MMR mutations are not the only contributing factor to cisplatin resistance and most of the human cell lines used to study cisplatin resistance contain multiple alterations. Most common is a dominant negative mutation in p53 in addition to the MMR mutation (60, 73). Nonetheless, there is an obvious interaction between mismatch repair deficiency and cisplatin resistance, although for human cancers this association is likely indirect and may involve mutations in several genes or repair pathways (4, 10, 60).

Deficiency in MMR leading to cisplatin resistance implies that MMR plays an active role in the cytotoxic ability of the drug. The mechanism is not known. Several studies, including those presented in this thesis, propose that MMR is involved in cisplatin lesion recognition. *E. coli* MutS as well as yeast hMSH2, and human MutS-alpha heterodimer, specifically bind to cisplatin 1,2-GG adducts *in vitro*, while none of these proteins binds with high affinity to any of the other cisplatin produced lesions or *trans*-isomer adducts (9, 28, 74, 75). The resulting binding affinity for these lesions is about 10-fold lower than a G-T mismatch (9). Several models have been proposed to explain how MMR can induce death in cells with DNA damaged by cisplatin, most of
which include MMR interaction or interference with other cellular pathways of repair, thus causing cell death, but the details are still unclear.

**Repair of cisplatin lesions by nucleotide excision repair.**

Nucleotide excision repair (NER) is both highly efficient and relatively nonspecific in terms of its ability to repair a variety of DNA damage. The basic damage signature for recognition by NER are distortions in the double helix. However, NER will not repair distortions from base mismatches, 8-oxoguanine or O6-methylguanine lesions. Other repair systems, such as those previously discussed, are primarily responsible for repair of such lesions. NER is rather efficient at repairing damage from UV- irradiation as well as bulky lesions caused by DNA exposed to drugs such as psoralen or benzo(a)pyrene. NER is also the primary mechanism for recognition and excision of cisplatin adducts from DNA (4, 50).

The basic steps of NER are well characterized in *E. coli* and are shown in Figure 1-5. Removal of 1,2- and 1,3- intrastrand cisplatin lesions and interstrand crosslinks is achieved by the NER holoenzyme, UvrABC, however, the 1,3- d(GpNpG) lesions are removed 50-fold faster than those between adjacent purines (76). A dimer of the UvrA protein and a dimer of the UvrB protein are bound nonspecifically to DNA, migrating up and down the helix, and arrest once the complex recognizes a distortion in the DNA, such as a cisplatin lesion. UvrB binds the damaged lesion, which subsequently causes UvrA
Figure 1-5. **Removal of damage by NER.**

The UvrABC endonuclease can remove 1,2- and 1,3-intrastrand crosslinks and interstrand crosslinks lesions. Nicks on both sides of the lesion (represented by the symbol) are introduced by the action of UvrC protein. UvrD helicase removes the incised piece of DNA and poll re-synthesizes the small gap. Adapted from (77, 78).

Dimers of UvrA and UvrB travel along the DNA and bind tightly once a lesion is encountered.

UvrC nicks the DNA first 3' to the lesion and then makes a subsequent nick 5' to the lesion, excising a small DNA fragment containing the lesion.

* Cho protein can also replace UvrC and nick 3' to the lesion but UvrC is still required for 5' incision.

Binding the lesion causes the dimers of UvrA and one monomer of UvrB to leave the damage site allowing UvrC to bind to UvrB.

Poll and ligase re-synthesize and seal the small gap to complete repair.
and one monomer of UvrB to be displaced by UvrC. The UvrBC complex nicks the DNA by allowing UvrC to make an incision in the DNA four nucleotides 3' to the damage site which induces another UvrC incision seven nucleotides 5' to the damage site. Once the DNA is cut, UvrD helicase removes the short piece of DNA which contains the damage, DNA polymerase I loads onto the DNA to re-synthesize the DNA using the complementary strand as a template, which is finally sealed by DNA ligase (77, 78). In addition, another recently identified protein, Cho (UvrC homolog), induced by the SOS response is also involved in NER. It shares homology with the N-terminal domain of UvrC, which is responsible for 3' incision activity. Cho can also bind to UvrB using a different binding domain in UvrB in place of the UvrC protein (78). Cho can only make the 3' incision and does so four nucleotides from the normal UvrC incision site (78). In order to complete NER repair, Cho must be replaced by UvrC at some point in the repair pathway, as it has no 5' incision ability. It is speculated that the role of Cho in NER is to excise lesions that are poorly excised by UvrC. Poor UvrC incision is probably due to geometric constraints caused by the lesion, which can be circumvented by the ability of Cho to bind UvrB at a different binding site than UvrA, which in turn gives better access to the damage site (78). Because Cho is induced by the SOS response, it may also participate in NER when a replication fork encounters a lesion that has not been acted on by UvrC or cannot be acted on for the explanation just given (78). Support for NER removal of cisplatin adducts is evident by sensitivity of NER mutants of *E. coli* and mammalian cells to cisplatin (10, 79). As a deficiency in NER causes cisplatin lesions to persist in the DNA, upon replication the fork will be blocked by the adducts.
The fork will likely stall or disintegrate, which can ultimately lead to double strand break formation. These DSBs must be repaired by recombination or cell death will ensue (10, 80).

**Repair of methylated bases by base excision repair.**

Methylating agents, such as MNNG, presented earlier, modify bases to produce lesions such as 3-methyladenine, 7-methylguanine and O⁶-methylguanine. The O⁶-methylguanine lesion in DNA can be removed directly by DNA-methyltransferases and is shown schematically in Figure 1-6 (18, 77). The mechanism of the methyltransferases is to displace the methyl group from the DNA base, in this case the O⁶-position on the guanine, to an internal site within the protein. Two methyltransferases can use the O⁶-methylguanine lesion as a substrate, the Ogt protein and the Ada protein. Ogt is important for actively growing cells and Ada takes over upon induction of the adaptive response. In order to demethylate a damaged DNA base, Ada protein will bind directly to the damaged base and flip it into its substrate binding pocket, out of the double helix structure, which allows transfer of the methyl group from the base to a cysteine residue located internally(18).

An important feature of the Ada protein is that it has the ability to act as a transcriptional activator for itself and other repair proteins. Its regulation is achieved through its methylation state and two internally located cysteine residues control its methylation status. Methylation at either site will prevent further acceptance of methyl
Figure 1-6. Direct removal of $O^6$-methylguanine adducts from DNA.

Methylating agents such as MNNG, can form of $O^6$-methylguanine lesions in the DNA. A methylguanine methyltransferase, such as the Ada protein, can directly remove the methyl group restoring the normal guanine base.
groups at that position, which makes Ada non-functional for further repair of methylation damage (18). The binding of methyl groups at the Cys38 residue converts the activity of Ada from a methyltransferase to that of a transcriptional activator, in a process termed the Ada or adaptive response. This enables Ada to upregulate transcription of itself as well as other genes involved in repair of methylation damage, thus enabling the cell to repair higher levels of damage from exposure to alkylating agents (18).

The Ada gene is part of an operon which also contains the *alkB* gene. AlkB primarily removes 1-methyladenine and 3-methylcytosine lesions from DNA and is upregulated when Ada increases its own transcription. The *aidB* and *alkA* genes which are also induced in response to Ada and are postulated to be activated by an increase in transcription from UP element binding to the promoters upon Ada reaching fully methylated conformation (50, 81). The product of the *aidB* gene is an isovalent-coA dehydrogenase and is postulated to aid in resistance of cells to methylation damage from reaction with nitrosamines, especially during stationary phase (82). AlkA encodes an N-glycosylase which has the ability to recognize a broad range of substrates including 3-methyladenine, which is readily produced by reacting MNNG with DNA (18). The O\textsuperscript{2}-methylcytosine, O\textsuperscript{2}-methylthymine, 3-methyladenine and 7-methylguanine lesions created by methylating agents are removed by base excision repair and which is shown in Figure 1-7. The base excision repair process uses glycosylases to repair damage as opposed to the methyltransferases just discussed. These enzymes bind to the site of damage, flip out the modified base and then break the bond between the base and the
Many agents modify DNA bases which are then recognized by glycosylases. Alkylated bases are removed by N-glycosylases, which flip the damaged base out of the DNA helix for excision. This leaves an AP site in the DNA which is cut by an AP endonuclease. Exonucleases degrade the strand and repair polymerases resynthesize the cut strand, which is sealed by ligase. In the case below 3-methyladenine is being removed by 3-methyladenine glycosylase.
deoxyribose leaving an AP (apurinic or a pyrimidinic) site (81). This is in contrast to the methyltransferase which also flips the base out of the DNA helix, but only excises the methyl group from the base not the entire nucleotide. These AP sites are in turn, recognized by AP endonucleases that cleave the DNA backbone leaving a 3'-OH and a 5'-deoxyribophosphate. A repair polymerase, attaches to the nick in the DNA and either using its own 5'-3' exonuclease or a different cellular exonuclease, removes a short stretch of DNA (5 nucleotides or less) that includes the AP site. The small gap is resynthesized and DNA ligase completes the base excision repair process (50). AlkB is different from both AidB and AlkA in that its mode of repair does not have methyltransferase, nuclease or glycosylase activity associated with it (83). AlkB can revert 1-methyladenine and 3-methylcytosine bases back to adenine and cytosine by oxidative demethylation (18). The AlkB protein function was discovered because of its resemblance to other α-ketoglutarate-Fe^{2+} dependent dioxygenases. It uses metal (Fe) catalysis to oxidize the methyl groups attached to the ring nitrogens of bases and is most effective acts on single-stranded DNA, as these lesions are less frequently produced in double strand DNA. The oxidized methyl group from the base is unstable and is spontaneously released as formaldehyde, which allows for restoration of the original base without complete excision of the base. Mutants of alkB are not as sensitive to damage from MNNG as the lesions it is most specific for are formed in low amounts (84). However, alkB mutants are much more sensitive to a different subset of alkylating agents such as the class which includes methyl methane sulfonate (MMS). Generally, bacteria mutant in base excision repair genes are more sensitive to methylating agents than wild
type cells, indicating their requirement for removal of methylation damage (83, 84).
Unrepaired AP sites are a block to polymerase progression (1), which again provides
another possible mechanism for double strand break formation in the cell.

**Replication fork collapse and recombinational repair of drug-induced lesions.**

The primary mechanism for removal of cisplatin adducts and methylation damage
is NER and BER, respectively. However, homologous recombination also plays a critical
role in tolerance of these lesions, especially when the primary repair mechanism is non-
functional. Even when repair systems are operative, residual levels of unrepaired lesions
are still present in the cell. This is where recombination is critical because the replication
fork can encounter lesions not yet reached by the repair systems, causing additional
damage which can only be repaired by recombination. Recombination deficient mutants
of *E. coli*, especially *recA* mutants, show a decreased survival upon exposure to cisplatin
and methylating agents, indicating their importance for repair of these lesions as well as
other damage (1, 80). Gene products necessary for recombinational repair of cisplatin
lesions will be presented in Chapter 2. As stated previously, cisplatin lesions are a direct
block to polymerase progression, so once the replication fork encounters such a lesion,
the fork can stall or disintegrate, resulting in lethality if the replication machinery does
not reload. Figure 1-8 outlines potential types of damage a replication fork can
encounter, all leading to a situation where recombination is invoked for cell survival (85).
The cisplatin scenario is presented in Figure 1-8 D and E, where the means by which
recombination is initiated is different depending on whether the lesion resides in
Figure 1-8. Potential types of replication fork damage.

The replication fork can encounter different types of damage which it can pass through, in the case of (a) or stall at (d,e) or collapse upon (b,c), invoking recombination for repair, as reloading the replication fork is essential for cell survival. Replication forks encountering cisplatin lesions on the lagging or leading strands are represented in (d) and (e).

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the leading or lagging strand. If the lesion is in the lagging strand, the effect on synthesis is minimal, as the polymerase will pause when the replication machinery encounters the lesion, but synthesis can reinitiate downstream of the lesion without incident. The result is formation of a gap of single stranded DNA containing the lesion, called a daughter strand gap (DSG) (10). DSGs opposite a cisplatin lesion are repaired by recombination using *recA*, *recFOR* and *polA* gene products, using an intact daughter strand template (10, 86). The process is outlined in Figure 1-9A. The recombination process allows the cell to tolerate the cisplatin lesion which can be removed from the DNA during or after recombination by NER.

Another mode of recombinational repair occurring with cisplatin damage is the double strand break (DSB), which can form in several ways. Blockage of the replication fork by the cisplatin lesion in the leading strand causes uncoupling of leading and lagging strand synthesis, followed by fork stalling (10). After stalling, the fork can regress, resulting in the newly synthesized stands forming a flush-ended duplex. The isomerized Holliday junction structure produced by the fork regression can be a substrate for RuvC, which can cleave the structure creating a DSB. Alternatively, a new fork can encounter the double strand ends, causing fork collapse and subsequent DSB formation. We presume this could be one mechanism to explain how cisplatin leads to DSB formation. There are a few natural mechanisms for DSB formation in the cell. Not all replication forks reach their final destination without interruption, and a percentage of forks will stall without reason or as a result of encountering normally occurring DNA damage (87).
Figure 1-9. Daughter strand gap and double strand break formation and repair (10).

Models for recombinational repair of DSGs and DSBs induced by cisplatin damage. (a) is DSG repair and (b) is DSB repair. After replication fork stalling at a lesion, the fork is repaired by DSG repair, which requires an intact homolog provided by multiple initiations of chromosome replication at the origin. DSBs formed require the RecBCD DSB repair pathway to restore an active fork. A cisplatin lesion in the DNA can be acted upon by the NER pathway during or post recombinational repair. ABP, adduct binding proteins can enhance the block to replication.
Either circumstance can cause a DSB if another fork encounters the already stalled/regressed fork, by the mechanism proposed above. Another instance where a DSB can form is with \textit{dam} mutants, where MutH incision can occur on either the parental or daughter DNA strands. MutH incision at two GATC sites directly opposite one another on the parental and daughter strands could lead directly to DSB formation in \textit{dam} mutants (45). In another case, if MMR has already initiated repair at a damage site, the gap created by MutH incision and the subsequent excision step of MMR, will cause fork collapse and DSB formation if a new fork encounters the repair in progress (88). With cisplatin, the circumstance for DSBs to arise is similar. If a replication fork encounters a pre-existing nick in the DNA, by MMR in progress, or from an unpaired DSG from previous initiation of repair at a cisplatin adduct, it can cause a DSB, created from fork collapse upon encountering the gap (10). Repair of DSBs requires the RecBCD recombinational repair pathway and is shown in Figure 1-9B. Once a fork has regressed the double strand ends created are a substrate for RecBCD action. RecBCD exonuclease action degrades the DNA and after encountering a Chi site, ultimately leaves a single stranded tail with a 3'‐end. This is now a substrate for RecA, which can load and promote strand exchange. Resolution of the Holliday junctions by RuvABC restores the fork and PriA pathway proteins can reload the replication machinery for re-start (86, 89). Replication restart is an integral part of DSB repair from fork collapse and now lesion has another opportunity to be acted upon by repair pathways. Initially once stalled, there is also the potential for the fork to isomerize into a cruciform structure to become a substrate for RuvC cleavage (90), as previously stated, leading to a DSB. It is known
that both DSG and DSB repair pathway proteins are required to survive cisplatin induced damage (10, 80).

Damage from methylating agents can have a similar effect when encountered by the replication fork. O\(^6\)-methylguanine lesions in DNA are all subject to MMR as their pairing with C or T are both recognized by MutS (21). As MMR uses the same polymerase as replication and MMR occurs just after replication, this theoretically offers another mechanism for stalling of the replication machinery. Futile cycling of the polymerase for repair of O\(^6\)-lesions could impede fork progression leaving it susceptible to reversal or disintegration requiring the same mechanisms for recombination to restore active replication. The details of this hypothesis and both DSG and DSB repair pathways in regard to cisplatin and methylation damage will be discussed in detail in the upcoming chapters.

**The role of mismatch repair and recombination in sensitizing cells to DNA damaging agents.**

The mismatch repair status of a cell directly affects the sensitivity of the cells to specific methylating agents and cisplatin. Using *E. coli* as a model system, it was found that bacteria mutant in the *dam* locus are more sensitive to cisplatin and MNNG than their isogenic wild type (21, 69). If an additional mutation was made in a mismatch repair gene such as *mutS* or *mutL* in combination with *dam*, sensitivity was restored to wild-type levels (21, 69). Thus, MMR is responsible for sensitizing cells to these types
of DNA damage. Human cells display a response to cisplatin and methylating agents similar to the *E. coli dam* mutant. Mutations in mismatch repair genes render human cells resistant to treatment with cisplatin and methylating agents (67, 91-93).

It is postulated that the mechanism for mismatch repair sensitization of *dam* bacteria to MNNG is due to the base pairing ability of O$^6$-methylguanine (21). During replication O$^6$-methylguanine can form base pairs with cytosine or thymine. Neither C nor T is a “good” base match for the O$^6$-methylguanine base and either will be readily recognized by the MutS protein. This leads to a futile cycling of recognition and excision by the mismatch repair process, diagrammed in Figure 1-10 (21). Re-synthesis during MMR allows PolIII to pair the O$^6$-methylguanine with a cytosine or thymine, neither of which is acceptable, causing MutS to continually initiate repair at the lesion. As PolIII resynthesizes the MMR created gaps, the constant excision process causes replicative PolIII replication fork stalling, leading eventually to cell death. The exact mechanism of this is not known and we postulate that DSBs resulting from fork disintegration are likely the cause. In the absence of MMR, the polymerase does not stall because there is no mismatch repair and, unless the lesion is removed directly, the O$^6$-methylguanine can persist in the DNA causing an increase in mutation rate, and providing the cell with the ability to avoid death. As stated previously, the exact mechanism of this and the resulting effect requiring recombination is still not understood. Futile cycling can also occur at sites away from the replication fork as a consequence of MMR recognition and MutH incision on unmethylated DNA at O$^6$-meG-C base pairs to promote DSB formation (141).
Figure 1-10. **Futile cycling at the replication fork.**

DNA polymerase III holoenzyme has placed a C opposite an O\(^6\)-methylguanine (G-Me) template residue. This base pair is recognized and acted upon by the MMR system. Replacement of the C with a T again results in a mismatched base pair subject to MMR. Because DNA polymerase III holoenzyme is required for repair synthesis, it is speculated that the replication fork stalls near the mismatch. In the absence of MMR, no stalling of the fork occurs.
It is the latter that occurs in \textit{dam} mutants, but not in wildtype, and may be principally responsible for MMR sensitization to MNNG. The situation with cisplatin is somewhat more complicated, in that there is no model for mismatch repair sensitization with this compound. It is known that the MutS protein can recognize both simple and compound cisplatin lesions and that recombination is as essential for cell survival of cisplatin damage as NER (10, 28). We postulate that during recombinational repair cisplatin lesions are recognized by MutS because they are perceived as a type of homeologous DNA. As DNA damage induced by exposure to cisplatin invokes recombinational repair, and as cisplatin lesions can be recognized by the mismatch repair system, it is likely that mismatch repair interferes with recombinational repair of cisplatin adducts. During RecA-mediated strand transfer with substrate(s) containing platinated adducts, we postulate that MutS will recognize the lesion, abrogating branch migration and heteroduplex formation. This rationale acts as a basis for a possible mechanism for cisplatin drug resistance, as well as the basis for work presented in this thesis to investigate MMR interference with recombinational repair of DNAs containing damage from drug lesions.
CHAPTER II

SPONTANEOUS AND CISPLATIN-INDUCED RECOMBINATION IN
ESCHERICHIA COLI

Abstract

To measure cisplatin-induced recombination, we have used a qualitative intrachromosomal assay utilizing duplicate inactive lac operons containing non-overlapping deletions and selection for Lac\(^+\) recombinants. The two operons are separated by one Mb and conversion of one of them yields the Lac\(^+\) phenotype. Lac\(^+\) formation for both spontaneous and cisplatin-induced recombination requires the products of the recA, recBC, ruvA, ruvB, ruvC, priA and polA genes. Inactivation of the recF, recO, recR and recJ genes decreased cisplatin-induced, but not spontaneous, recombination. The dependence on PriA and RecBC suggests that recombination is induced following stalling or collapse of replication forks at DNA lesions to form double strand breaks. The lack of recombination induction by trans-DDP suggests that the recombinogenic lesions for cisplatin are purine-purine intrastrand crosslinks.
Introduction

Cisplatin (cis-diaminodichloroplatinum (II)) was discovered through its ability to inhibit cell division in *Escherichia coli* after its production during electrolysis from platinum electrodes (94). The drug also effectively inhibits growth of mammalian cells in culture (95) and it is currently used for the treatment of a variety of cancers particularly those occurring in the testicles where the rate of cure is greater than 90% (3). In spite of intensive efforts, the molecular mechanism leading to cell death after cisplatin treatment is not known.

Cisplatin reacts with DNA to produce mostly intrastrand crosslinks between adjacent guanines (65% of the total), adjacent guanine and adenines (25%), and guanines separated by a base (1,3-GNG, 5-10 %) (6, 8). Interstrand crosslinks comprise about 2% of the total adducts. In contrast, the biologically inactive *trans* isomer (*trans*-diaminodichloroplatinum (II), *trans*-DDP) produces mainly adducts between guanines separated by a base (40%) and interstrand crosslinks (20%) (6, 15). Both cisplatin and *trans*-DDP produce small amounts of monoadducts. These determinations suggest that intrastrand crosslinks between adjacent purines are the biologically important adducts since they efficiently block progression of DNA polymerases *in vitro* and *in vivo* (14). Nucleotide excision repair (NER) removes intrastrand crosslinks but the 1,3-GNG lesions are removed at a rate 50-fold faster than those between adjacent purines (76). The
importance of NER is manifested by the increased sensitivity of NER-deficient mutants of *E. coli* (91) and mammalian cells to cisplatin but not *trans*-DDP (4, 92). Transcription-coupled repair is more efficient than global repair since removal of adducts from actively transcribed DNA is faster than from non-transcribed DNA (96). In addition, however, recombinational repair mechanisms are as important as NER in allowing cells to survive cisplatin damage (10, 79).

The major pathway for recombination in *E. coli* in conjugational crosses is dependent on RecBCD (86, 97, 98). This enzyme binds to blunt-end DNA molecules and moves along the DNA using its helicase and 3'-exonuclease activities. When the enzyme encounters an eight base Chi sequence, the enzyme continues to unwind DNA but its exonuclease specificity alters to favor 5'-end degradation. The encounter with a Chi site leads to modification of the RecD subunit thereby allowing polymerization of RecA on the 3'-strand and subsequent strand invasion of a homologous DNA duplex to initiate recombination. In the absence of RecBCD, recombination can occur by the RecF or RecE pathways. The RecE pathway proteins are encoded by genes on a prophage (86, 97) but since it is not present in the strains used here, this pathway will not be discussed further. The RecF pathway uses the RecFOR proteins to load RecA onto DNA, particularly at gaps. The RecFOR complex can substitute for RecBCD functions but only if SbcB and SbcDC nucleases are inactivated. The RecF pathway genes, in addition to *recFOR*, comprise *recJ, recN* and *recQ*. RecJ is a 5' to 3' nuclease and RecQ has helicase activity. The biochemical activity associated with RecN is not known. Although the RecBC and
RecF pathways have different substrates, they both require RecA and downstream proteins such as the RecG and RuvAB Holliday junction helicases and the RuvC resolvase.

It has been shown previously that recombination-deficient strains belonging to the RecBC and RecF epistasis groups show decreased survival to cisplatin, but not trans-DDP, compared to wildtype (10, 79). These included \textit{recBCD}, \textit{recFOR}, \textit{ruvABC} and \textit{recG} but not \textit{recN} or \textit{recQ}. It was concluded that cisplatin DNA damage included the formation of both double-strand breaks (DSBs) and gaps that required recombinational mechanisms for their repair. Furthermore, we showed that \textit{recBC} cells are as sensitive to cisplatin as NER-deficient (\textit{uvrA}) bacteria and that the double mutants (\textit{uvrA recBCD} or \textit{uvrA recF}) are much more sensitive than either alone (10). This indicates that recombinational mechanisms are as important as NER for survival against cisplatin challenge. Although the survival data for recombination-deficient bacteria are indicative of double-strand break and gap recombinational repair, the detailed mechanism(s) by which recombination enhances survival has yet to be defined.

The relationship between DNA replication and recombination has been clarified in recent years (89, 90, 98, 99). Replication forks can stall at natural or damaged DNA sequences which may result in the formation of regressed replication forks that become substrates for RuvAB or RecBCD. Several outcomes are possible, including cleavage of the regressed fork by RuvC, followed by recombination or initiation of RecBCD-
dependent recombination if a Chi sequence is encountered while unwinding the regressed fork. Replication fork collapse can occur if the fork encounters a nick or gap in DNA leading to formation of a DSB that requires RecBCD-dependent recombination for its repair. A consequence of fork stalling or collapse is dissociation of the replicative DNA polymerase holoenzyme complex from the fork. During or after restoration of the fork, it is necessary to re-assemble the holoenzyme and this requires enzymes in the PriA-dependent initiation of chromosome replication pathway (100). PriA can bind to D-loops that are formed as recombination intermediates and is able to block DNA polymerase extension and to recruit DnaC protein to begin holoenzyme assembly (101). Induction of DNA damage by ultra-violet light leads to the formation of replication-blocking lesions that can result in fork regression (102). The reversed fork is thought to be stabilized by RecA and RecF to prevent degradation by RecQ helicase and RecJ nuclease (103). These proteins, therefore, are not participating in a traditional recombination event even though RecA is probably mediating fork reversal.

Antitumor agents such as cisplatin frequently induce genomic instability and rearrangements in populations of cells exposed to them (1, 104-106). Indeed, the ability of radiation to promote mitotic recombination has been known since early last century and small doses of ultraviolet light are frequently used in phage and bacterial genetics to stimulate recombination. The molecular basis for this phenomenon is still not entirely clear but the formation of DSBs is suspected to be the initiating event (107). We showed previously that cisplatin, but not trans-DDP, was highly recombinogenic in E. coli using
an assay system developed by Konrad (108). We have extended this observation by determining the genetic requirements for cisplatin-induced recombination. We show that the RecBC and RecF pathway gene products are required.
Materials and methods

Bacterial strains, plasmids and media: The *E. coli* K-12 strains used in this study are listed in Table 1. The genotype of strain GM7330 has recently been modified by the *E. coli* Genetic Stock Center. The *lacMS286* deletion is now designated as *(lacY-lacZ)286* and the *phi80dIILacBK1* deletion as *lacZ9*. Strains were constructed by P1*vir* transduction. Plasmid *precA430*, which expresses the wildtype RecA protein under control of the *tac* promoter in a pBR322 backbone (109), was supplied by Dr. Kendall Knight (UMass Medical School, MA, USA). Plasmid pCDK3, with the cloned *argA*, *recB*, *recC* and *thyA* genes (110) was obtained from Dr. S.R. Kushner (University of Georgia, GA, USA). Plasmid pGB2-*ruvAB* (90) was a gift from Dr. Benedicte Michel (INRA, Jouey-en Josas, France). MacConkey Agar (Difco) medium was supplemented with additional 10 gm/l lactose before plate preparation. Strain GM7330 and its derivatives were grown overnight in L medium and diluted ten- or one hundred-fold in minimal salts. Three ml of diluted cells were placed on the surface of a MacConkey agar plate, allowed to settle for 10 min and then the excess medium removed by aspiration. This procedure ensured a uniform lawn of cells on the plate. Sterile 6.35 mm disks were placed on each dry plate and aliquots of cisplatin (1, 2 and 4 µl of a 1.2 mg/ml solution) added to the disks and allowed to dry. The plates were incubated for 48 hr at 37º C. Plates were scanned with an Epson Perfection 1250 scanner and the images managed with Adobe Photoshop software.
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<td>Lab stock</td>
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<sup>a</sup>CSC, *E. coli* Genetic Stock Center, Biology Department, Yale University, New Haven CT 06520-8103, USA

<sup>b</sup>plus unidentified suppressor mutation allowing growth on rich medium
L broth consists of 20 gm tryptone, 10 gm yeast extract, 1 gm NaCl and 4 ml 1 M NaOH/I, solidified when required with 16 gm agar (Difco). Minimal salts solution was that described by Davis and Mingioli (111). Chloramphenicol, kanamycin, ampicillin, spectinomycin and tetracycline were added to media at 10, 20, 100, 50 and 10 μg/ml respectively.

Cytotoxicity assay: Overnight cultures were diluted 100-fold into fresh L medium and grown to a density of about $10^8$ cells per ml. The cells were centrifuged and resuspended in an equal volume of minimal salts and treated with cisplatin for 60 min. The cisplatin (Sigma) was dissolved in water for 2 hrs at 37°C before use. The treated cells were diluted and plated on L medium to measure survival.
Results

Lac\(^{+}\) recombinants arise by conversion of lacZ\(^{9}\).

In the assay system developed by Konrad (108), one defective lac operon ((lacY- lacZ)\(^{286}\)) is at its normal chromosomal location and the other (lacZ\(^{9}\)) is part of a defective phi80 prophage (Figure 2-1). To determine which of these is converted to the Lac\(^{+}\) phenotype, ten spontaneous and ten cisplatin-induced Lac\(^{+}\) recombinants were isolated from strain Hfr KS391. These were then mated for either a short or long period with a recipient that was Lac\(^{-}\), due to the presence of a lac-pro deletion, and Trp\(^{-}\) due to insertion of a Tn10 element. Recombinants arising from the short mating and Pro\(^{+}\) [Trp\(^{-}\)] selection were all Lac\(^{-}\). On the other hand, Lac\(^{+}\) recombinants arising from the longer mating and Trp\(^{+}\) [Pro\(^{-}\)] selection were all Lac\(^{+}\). This result indicates that conversion occurred in the lac region carried by the phi80 prophage in both spontaneous and cisplatin-induced Lac\(^{+}\) recombinants.
Figure 2-1. **Recombination assay.**

Strain GM7330 contains two copies of the *lac* operon, each of which is inactive due to a deletion mutation. Cross-overs, as shown, can generate an active *lac* operon.
recA and recBCD mutants are recombination-deficient.

A typical result with the wildtype strain, GM7330, is shown in Figure 2-2a. Four filter paper discs are placed on a lawn of bacteria on MacConkey medium and various amounts of cisplatin are added to the discs. After 2 days incubation, the plates show a uniform distribution of spontaneously-arising Lac\(^+\) colonies on a background of Lac\(^-\) colonies. Around the disks containing cisplatin, a dose-dependent increase in Lac\(^+\) recombinants is observed indicating drug-induced recombinants. Given the limitations of the assay and the large number of mutants to screen, we have not attempted to quantitate the result for each strain but rather to obtain a qualitative positive or negative score. The data for every individual strain are not shown as the wildtype or recombination-deficient responses are almost identical in the different mutants.

The RecA protein promotes homologous pairing and strand exchange (86, 97, 98) and so it was not unexpected that no spontaneous or cisplatin-induced Lac\(^+\) recombinants were found with strains mutant for recA\(^56\) and a recA deletion (Figure 2-2c). Relative to wildtype, there was a pronounced dose-dependent clear zone surrounding each disk containing cisplatin indicating increased killing with this compound. The recA mutant strains harboring a recA\(^+\) plasmid had a wildtype level of recombination (Figure 2-2d). The RecBCD complex has several enzymatic activities including helicase and exonuclease activities and is responsible for loading RecA onto DNA at Chi sites. Only a few Lac\(^+\) recombinants were detected in a recBCD deletion strain and increased
Figure 2-2. **Lac\(^+\) recombination in wildtype and mutant derivatives of GM7330.**

(a) Sporadic red spontaneous Lac\(^+\) recombinants uniformly cover the surface of the plate on a background of Lac\(^-\) colonies of the wildtype strain, GM7330. Four circular filter paper disks are present on the surface of the plates that contain increasing amounts of cisplatin in a counterclockwise direction (1, 2 and 4 mM). The disc at the top of the plate did not contain cisplatin. (b) The wildtype strain response to trans-DDP. (c) Recombination-deficiency of the ΔrecA-srl strain, GM7661. (d) Complementation of recombination-deficiency in GM7661 by plasmid precA430. (e), (f), (g) Recombination-deficiency of strains GM7346 (ΔrecBCD), GM7390 (ΔruvABC) and GM7649 (ΔpolA). (h) Complementation of GM7649 recombination-deficiency by F\(^-\)-polA\(^+\) (i) Response of GM7769 (ΔrecF) (j) Response of GM7382 (recJ) (k) Response of GM7368 (uvrA6) (l) Response of GM7368 (uvrA6) to trans-DDP.
cytotoxicity to cisplatin was evident (Figure 2-2e) as previously reported (10, 91). In contrast, Lac$^+$ recombinant formation in a recD mutant strain, which lacks 5'-exonuclease activity, was indistinguishable from wildtype (data not shown). The recombination-deficiency of the recBCD mutant was restored to the wildtype level by complementation with plasmid pCDK3 which carries the recBCD wildtype genes (data not shown).

**ruvABC strains are recombination-deficient.**

RuvAB helicase binds specifically to Holliday junctions and promotes translocation of such junctions. The RuvC protein binds specifically to a Holliday junction and cleaves it in one of the two possible orientations (86, 97, 98). No spontaneous or cisplatin-induced Lac$^+$ recombinants were formed with ruvA::Tnl0 (polar effect on ruvB, data not shown), ruvC (data not shown) or ruvABC deletion mutants (Figure 2-2f). Increased sensitivity to cisplatin was evident from zones of clearing around the disks confirming previous data. The suppression of recombination is in contrast to results obtained in conjugal and transductional crosses in which ruvABC mutant alleles show little effect on recombination proficiency (112). The combination of a recG mutant allele with any ruv mutation, however, causes recombination deficiency in such crosses although recG mutations alone have no effect (112). In the lac assay, the recG mutant behaved as wildtype (data not shown) and ruv recG double mutants were as deficient for Lac$^+$ formation as the ruv single mutants or the ruvABC deletion (data not shown).

Introduction of plasmid pGB2-ruvAB into the ruvA::Tnl0 strain resulted in wildtype level
of Lac recombinants (data not shown). These results indicate that in the lac assay, RuvABC is required for junction translocation and cleavage.

**polA strains are recombination deficient.**

Mutant strains deleted for the polA gene grow on minimal but not on rich medium indicating a requirement for polymerase I at high growth rates (113). On rich medium, however, suppressor mutations occur at high frequency that allow growth of polA deletion mutants (113). We transduced the polA deletion mutation into the wildtype GM7330 to produce GM7647 and then isolated a derivative that was able to grow on rich medium (GM7649). Both GM7647 and GM7649 were very sensitive to methyl methane sulfonate (108), a characteristic trait of polA mutants. Only GM7649 was able to grow well on MacConkey medium but it did not produce Lac+ recombinants and it showed increased sensitivity to cisplatin (Figure 2-2g). The survival of the polA mutant in liquid medium after challenge with cisplatin is on the same order of magnitude as a recA deletion mutant (Figure 2-3) which is greater than that for the polA1 allele(91). We introduced an F plasmid bearing the polA+ gene into GM7649 and it was resistant to methyl methane sulfonate and formed Lac+ recombinants at the wildtype frequency (Figure 2-2h). This complementation indicates that it is the polA mutation and not the suppressor mutation that causes recombination-deficiency and cisplatin sensitivity. These results indicate that spontaneous and cisplatin-induced Lac+ recombination requires the participation of DNA polymerase I.
**priA mutants are recombination deficient.**

PriA protein is required for the *in vitro* initiation of DNA replication of phage phiX174 where it functions with other primosomal proteins to facilitate the loading of DnaB, the replicative helicase (114). Subsequently it was shown that although *E. coli priA* mutants are viable, they were sensitive to ultraviolet light, recombination-deficient and are induced for the SOS response (100). Based on the finding that PriA can initiate primosome assembly on recombination intermediates, it is currently thought that PriA acts in replication restart at stalled or collapsed replication forks (101). Like the *polA* deletion mutants, *priA* strains grow best on minimal rather than rich medium (100, 101).

We found that a *priA* deletion mutant was able to grow on MacConkey medium but failed to produce spontaneous or cisplatin induced Lac⁺ recombinants (data not shown). Increased sensitivity to cisplatin was evident from zones of clearing around the disks and this was confirmed by measuring survival in liquid medium (Figure 2-3). The *priA2* mutants are much more stable in an *sfiA* genetic background due to SOS induction (100, 101) and such a double mutant was used to measure survival as shown in Figure 2-3. The *sfiA* mutation, however, imparts resistance to cisplatin (see below) and the survival of a strain with only *priA2*, therefore, would probably be much less than that for the double mutant shown in Figure 2-3.
Figure 2-3. **Survival of wildtype and mutant strains after exposure to cisplatin.**
**recFOR and recJ mutants show decreased cisplatin-induced recombination.**

In the absence of RecBCD, recombination in conjugal crosses relies on the RecF ensemble of proteins which include the products of the *recF, recO, recR, recJ* and *recQ* genes (86, 97, 98). RecFOR proteins load RecA at gaps in DNA and RecJ is a 5' to 3' exonuclease. RecQ is a helicase and the founding member of a family of helicases, some of which have been implicated in human disorders such as Bloom's and Werner's syndromes (115). In the *lac* assay, the *recF, recO, recR,* and *recJ* mutants behaved identically including three different *recF* alleles: the insertion allele *recF322::Tn3* and two deletions, *recF::Tet* and *recF::Kan*. In the standard assay, these mutant strains showed the normal level of spontaneous Lac⁺ recombinant formation but there was severely decreased cisplatin-induced recombination (Figure 2-2i, j). Unlike the *recFOR* mutants, the *recJ* strain was resistant to the cytotoxic action of cisplatin (Figure 2-2i, j). The magnitude of the reduction in drug-induced recombination varied from experiment to experiment in these RecF pathway mutants and in some cases very few drug-induced Lac⁺ colonies were observed. At present, we do not know the origin of this variability.

**Other mutant alleles.**

We have tested mutant alleles of the following genes in the *lac* recombination assay: *dam, fpg, lexA* (Ind-), *mutH, mutL, mutS, mutY, recD, recN, recQ, sbcDC, sfiA, ung, uvrA* and *uvrD*. All were recombination proficient for spontaneous and induced Lac⁺ recombination at the wildtype level except for the *dam* strain where spontaneous Lac⁺
recombination was increased (data not shown). This result was expected as the lac assay was originally developed to select for mutants with a hyper-recombination phenotype among which were dam mutants (116). The result for the uvrA6 mutant, as an example of this group, is shown in Figure 2-2k.

The effect of trans-DDP.

We have tested the ability of trans-DDP to promote recombination on all the mutant strains described above at the same molar concentrations as cisplatin. In none of the strains was there a significant response and the results for two representative strains, the wildtype and uvrA6 mutant respectively, are shown in Figures 2-2b,l.

sfiA mutations increase resistance to cisplatin.

During the course of this work, we noticed that strains carrying mutations in sfiA were more resistant to the cytotoxic effect of cisplatin than the isogenic parent. The sfiA gene is transcribed as a member of the inducible SOS response and the gene product inhibits cell division leading to formation of long filaments (117). To quantitate the cytotoxic effect we measured survival of the sfiA mutant and its otherwise isogenic wildtype strain in liquid medium (Figure2-3). The data confirm the results in the plate assay that sfiA confers resistance to cisplatin. We assume that the basis for this phenomenon in Sfi+ strains is "lethal filamentation" arising as a consequence of SOS induction provoked by cisplatin. Lethal filamentation was first described as a response to ultraviolet-irradiation in E. coli B (118).
*recN* and *recQ* mutations increase cisplatin sensitivity in a recBCD background.

The *recQ* gene encodes a helicase that functions with the RecF epistasis group gene products (86, 97, 98). The function of the *recN* gene product is unknown but this gene is part of the SOS regulon and highly expressed after DNA damage (86, 97, 98). During the *lac* recombination studies with MacConkey plates we noticed that *recBCD recN* and *recBCD recQ* strains appeared to be more sensitive to cisplatin than *recBCD* bacteria. The data in Figure 2-4 confirm that the same holds true in liquid media. We interpret these data as previously suggested for ultra-violet light induced damage; that is, in the absence of RecBCD, some of its functions are substituted for by RecQ and RecN (97). RecQ might substitute for the helicase activity leaving exonuclease action or the prevention of RecA loading as possible functions for RecN.
Figure 2-4. Survival of *recN* and *recQ* cells after cisplatin exposure.
Discussion

Classical studies of *E. coli* recombination have utilized conjugation and transduction in which fragments of double-stranded DNA are introduced into recipient cells (86, 97, 98). In contrast, recombination in the *lac* system occurs in a covalently-closed circular molecule and double-stranded ends need to be generated in order to initiate recombination. Additional or alternative genetic requirements in the *lac* assay are, therefore, expected compared to those for conjugation and transduction where the donor DNA is linear. The *lac* recombination assay was designed by Konrad (108) to isolate hyper-recombination mutants and he showed that mutations affecting DNA ligase, DNA polymerase I, UvrD helicase and deoxyuridine-triphosphatase stimulated recombination. Although the assay as used here is qualitative in its nature, it is sensitive enough to detect these hyper-recombination mutants and those with a recombination-deficiency. The inverse orientation of the *lac* operons on the chromosome predicts that inversions should be major recombination product. These have never been detected, suggesting that inversion of the chromosomal region between the *lac* operons is a lethal event.

We have shown that the following enzymes are needed for spontaneous gene conversion to occur: RecA, RecBC, RuvABC, PolA, and PriA (Table 2-2). The dependence on RecA was expected as both RecBC and RecF recombination pathways in *E. coli* require this
Table 2-2. **Summary of mutant responses to cisplatin exposure.**

A "+" sign under recombination indicates a wildtype response and a "−" sign recombination-deficiency. A "+/-" indicates a variable reduced response. In the last column a "+" sign indicates sensitivity to cisplatin and "−" resistance. The sensitivity of the *dam* mutant was not always obvious under the *lac* assay condition on plates although the strain is sensitive in liquid medium.

<table>
<thead>
<tr>
<th>Mutant strain</th>
<th>Recombination</th>
<th>Sensitivity to cisplatin treatment</th>
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<tbody>
<tr>
<td></td>
<td>Spontaneous</td>
<td>Cisplatin-induced</td>
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<tr>
<td><em>recA</em></td>
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<td><em>lexA (Ind</em>)</td>
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<td><em>dam</em></td>
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protein. RecBCD acts on double-stranded ends suggesting that these are formed in the covalently-closed circular chromosome to initiate the recombination process. The involvement of PriA suggests that replication fork stalling or collapse may occur, and if so, fork regression could form the double-stranded ends required for RecBCD action (Figure 2-5). Our results extend those of Zieg et al (119, 120) who found a dependence on RecA, RecBC, DNA ligase, the Rep helicase and LexA in the lac assay. The conflicting results with the lexA mutant may be due to different culture conditions or genetic backgrounds.

The dependence of lac gene conversion on RuvABC and PolA contrasts with that for conjugal recombination. In the latter system, the polA deletion mutation has no measurable effect (data not shown) and ruvABC mutations have only a minor effect on conjugal recombination unless recG is also inactivated (112). Clearly only RuvABC is required for lac gene conversion and RecG is not. The requirement for PolA probably indicates that DNA synthesis in the gene conversion event occurs over a short region(s) that is not a substrate for other polymerases. The latter conclusion is also based on the recombination proficiency phenotype of the lexA (Ind-) mutant in which the genes for the translesion polymerases are repressed (117). Furthermore, the polA cells surrounding the cisplatin-impregnated disks should be fully induced for the SOS response and expressing the SOS-dependent translesion polymerase genes including polB. The lack of a recombinational response under these conditions indicate that PolII cannot be involved although polB mutants are sensitive to cisplatin (121). Generally, polymerase action in
Figure 2-5. **Model for replication fork stalling and reversal at a cisplatin DNA adduct on the leading strand.**

The replication complex stalls at a cisplatin adduct leading to fork reversal. After adduct removal the double-stranded ends of the newly-synthesized strands become substrates for RecBCD. The regressed fork can isomerize as shown in the middle of the figure. If RecBCD encounters a Chi site during digestion, the pathway on the left of the figure is followed. If RecBCD does not encounter a Chi site, the sequence on the right side of the figure ensues. The replication complex is reloaded onto the restored fork by the PriA pathway enzymes.
recombination models is to extend the 3'-end of the invading strand at a D-loop and in DSB repair to extend the complementary strand as well \((86, 97, 98)\). Given that the processivity of PolII is about 25 nucleotides and that the minimal gap size for polymerase III holoenzyme action is about 500 nucleotides \((122)\) it is reasonable to assume that the amount of DNA synthesis is less than 500 nucleotides per strand.

The origin of spontaneous Lac\(^+\) recombinants is unknown. The genetic requirements described above suggest the occurrence of DSB repair associated with chromosome replication. About 20% of replication forks in \(E. coli\) fail to reach their destination \((87)\) and the stalling or collapse of these forks may lead to DSBs. Fork stalling or collapse could occur at various types of spontaneous DNA damage that, when processed, could initiate recombination. The wildtype level of Lac\(^+\) recombination in \(ung, fpg (mutM),\) and \(mutY\) mutant strains suggests that lesions recognized by the glycosylases encoded by these genes are not responsible, although we have not yet tested combinations of these mutations. The data in the Results section exclude contributions by NER, mismatch repair or SOS induction to the initiation of spontaneous recombination.

In contrast to spontaneous Lac\(^+\) formation, cisplatin-induced recombination has a partial requirement for the RecF pathway of recombination in addition to RecBCD (Table 2-2). The contrasting results with cisplatin and \(trans\)-DDP indicate that dipurine intrastrand crosslinks are the most probable offending lesions stimulating recombination. That these adducts efficiently block progression of DNA polymerases and that Lac\(^+\) gene conversion requires PriA protein suggests that recombination initiation is dependent on
DNA replication. This could occur either by replication fork stalling at a cisplatin lesion in the leading strand followed by fork regression (90) (Figure 2-5) or daughter-strand gap repair (77) at a cisplatin lesion on the lagging strand (Figure 2-6) or both. The requirement for both the RecBCD and RecF recombination pathways in cisplatin-induced recombination is consistent with these possibilities and in each case, an increase in recombination is expected. The RecBCD pathway is used to overcome replication fork problems on the leading strand (Figure 2-5) while the RecF pathway is used to repair gaps in the lagging strand (Figure 2-6).

Our previous results (10) showing a greater sensitivity to cisplatin of recBCD mutants than recFOR mutants is also consistent with this hypothesis. However, the substantial reduction in recombination observed in the recFOR and recJ strains may indicate a role for these gene products in processing of blocked replication forks in addition to their role in gap repair. Indeed, Courcelle et al (103) have presented such evidence in the case of ultra-violet light-induced damage. An additional alternative is that certain cisplatin lesions in DNA require the simultaneous action of both recombination pathways as described for the repair of palindromes (123) and recombination of phage P22 transducing fragments (124).

The demonstration that regressed fork structures can be cleaved by RuvABC to produce double-strand breaks (90) raises the question of how such breaks arise after cisplatin damage. At present we do not know if RuvABC cleavage of cisplatin-induced
Figure 2-6. Model for recombinational repair of daughter-strand gaps.

Daughter-strand gaps are formed after the replication complex encounters a cisplatin lesion on the lagging strand (77) and are repaired by the RecF pathway.
regressed forks occurs. Mutation in the \textit{ruv} genes imparts a high degree of sensitivity to cisplatin (10) which reflects an essential role in recombinational repair but this does not exclude an additional role in cleavage of regressed forks. Alternatively, double-strand breaks could arise by collapse of replication forks encountering discontinuities in the DNA due to cisplatin adduct removal by NER (10). We are presently testing if, indeed, DSB formation occurs after cisplatin exposure and, if so, is it dependent on RuvABC and ongoing DNA replication.

We have divided the mutant strains we have tested into six groups on the basis of sensitivity to cisplatin and recombination response (Table 2-2). There are three groups of specific interest of which the \textit{recFOR} mutants were discussed above. The second group comprises the \textit{recJ} mutant which, although in the RecF pathway, is distinguished by its relative resistance to cisplatin compared to \textit{recFOR} mutants. This may be the result of multiple exonucleases in the cell that can substitute for each other. The third group includes \textit{uvrA}, \textit{recG} and \textit{lexA}(Ind) which although sensitive to cisplatin show wildtype levels of recombination. This suggests that their role is in repair and unrelated to recombination and is clearly the explanation for the \textit{uvrA} and \textit{lexA}(Ind) strains. The role of \textit{RecG} may be in resolving stalled forks without breakage as proposed by McGlynn and Lloyd (85).

We have suggested previously (10) that cisplatin exposure leads to formation of both single-strand gaps and double-strand breaks in DNA and that RecF and RecBCD
recombination pathways respectively are required for tolerance of cisplatin adducts. It is possible that although the RecBC pathway is primarily responsible for repairing DSBs and eliciting recombination at specific adducts, occasionally the RecF pathway takes over this function in stressed cells at the same or different adducts which have not yet been acted upon by RecBCD. In this model, the amount of drug-induced recombination would reflect the combination from both pathways. That RecN (a member of the SMC family of proteins (125)) and RecQ are required for viability after cisplatin challenge in recBC mutants (Fig. 4) supports this idea. The variability we found with the RecF pathway mutants can also be explained by this model.

Acknowledgements

We thank all those investigators listed in Table 2-1 for donating bacterial strains and plasmids and Wendy Matthews for constructing some of the strains. This work was supported by grants GM63790 (M.G.M) and CA86061 (J.M.E.) from the National Institutes of Health.
MUTS INHIBITS RECA-MEDIATED STRAND EXCHANGE WITH PLATINATED DNA SUBSTRATES

Abstract

Human cell lines and *Escherichia coli dam* mutants are sensitive to the cytotoxic action of the anticancer agent, cisplatin. Introduction of mutations disabling DNA mismatch repair (MMR) into these cell lines renders them resistant to the action of this drug. We used RecA-mediated strand exchange between homologous phiX174 molecules, one which was platinated and the other unmodified, to show that strand transfer is decreased in a dose-dependent manner. Transfer was severely decreased at 10 adducts per molecule (5386 bp) and abolished with 24 adducts. At low levels of adduction, addition of MutS to the reaction further decreases the rate and yield in a dose-dependent manner. MutL addition was without effect even in the presence of MutS. The results suggest that although MMR is beneficial for mutation avoidance, its antirecombination activity on inappropriate substrates can be lethal to the cell.
E. coli cells mutant at the dam locus have decreased amounts of Dam methyltransferase which affects the physiology of the cell in a variety of ways including mismatch repair (MMR) directionality (30). In wildtype cells, the MMR complex assembles on hemi-methylated DNA trailing the replication fork and includes the MutS protein which recognizes a variety of base mismatches and insertion/deletions; the MutH protein whose latent endonuclease activity is activated in the ternary complex and MutL which acts as a bridge between the two proteins (126). MutH binds preferentially to hemi-methylated DNA and introduces a nick 5' to the G in a -GATC- sequence in the newly-synthesized unmethylated strand of hemi-methylated DNA. The -GATC- sequences are also the substrate for Dam methyltransferase and fully Dam methylated DNA is resistant to MutH action.

In dam mutants, the directionality of MMR is lost and MutH incision can occur at -GATC- sequences in either the parental or daughter strands (30). Unlike the wildtype where MMR action is restricted to the hemi-methylated DNA trailing the fork, in dam mutants MutH incisions can occur at unmethylated -GATCs- anywhere in the chromosome. The presence of MMR-induced nicks or gaps results in the formation of double-strand breaks (DSBs) that require recombination to restore genomic integrity.
Inactivation of recombination ability by mutation in various genes leads to a lethal phenotype in *dam* mutants (88).

*E. coli dam* mutants are more susceptible to the cytotoxic action of cisplatin, an antitumor drug, than wildtype (69). Inactivation of MMR, however, results in near wildtype levels of drug resistance. That is, MMR action provokes cell death in *dam* bacteria exposed to cisplatin presumably through the recognition of cisplatin intrastrand cross-links by MutS (9). Mammalian cell lines also show sensitivity to cisplatin and MMR-deficient lines derived from them are resistant (67, 73), although whether resistance is due specifically to MMR-deficiency has recently been challenged (62). Cisplatin-resistant cells isolated from patients treated with this drug have also been shown to be deficient in MMR (67). Like the bacterial MutS protein, the human MutS-alpha counterpart also binds to cisplatin intrastrand crosslinks (74). At present the molecular mechanism of MMR-mediated drug resistance in *dam* bacteria and mammalian cells is not known.

In addition to preventing mutations (mutation avoidance), MMR in *E. coli* prevents recombination between similar, but not identical (homeologous), DNA sequences (52). Genetic crosses between *E. coli* and the closely-related *Salmonella typhimurium* are sterile unless the recipient bacterium is MMR-deficient in which case the recombination frequency is increased by at least 1000-fold to form chimeras containing genetic material from both organisms (52). Biochemical experiments
employing the closely related bacteriophages M13 and fd, showed that MutS and MutL blocked RecA-mediated strand transfer of homeologous (M13-fd) but not homologous (M13-M13) DNA substrates (54, 56). ATPase-deficient MutS proteins that can still bind to mismatches also inhibit the reaction with homeologous substrates (127).

Cisplatin-induced DNA damage in E. coli is repaired by nucleotide excision repair (NER) and recombination, both systems being equally important (10, 75). Given that any impairment of recombination ability in dam bacteria is expected to be lethal and that MMR blocks homeologous recombination, we hypothesized that recombinational repair of platinated DNA is functionally identical to homeologous recombination. To test our hypothesis we have used the RecA-catalyzed strand transfer reaction to show that MutS decreases transfer with platinated but not unmodified substrates.
Materials and Methods

Cell survival: Survival after exposure to cisplatin was performed as described previously (10, 69). Strain MV1161 has the genotype thr-1 araC14 leuB6 (Am) DE (gpt-proA)62 lacY1 txs-33 supE44 (AS) galK2 (Oc) hisG4 (Oc) rfbD1 mgl-51 rpoS396 (Am) rpsL31 (Str<sup>R</sup>) kdgK51 xylA5 mtl-1 argE3 (Oc) thi-1 rfa-550 and strain MV3855 has the following additional mutations: uvrA6 alkA1 tagA1 zhb::Tn5. Both strains were obtained from M. Volkert (UMass Medical School, Worcester, MA).

Proteins and DNA: RecA protein was purified from strain GM7487 (preCA430/F-lac<sup>P</sup> lacZΔM15 pro A<sup>+</sup>B<sup>+</sup>/ P90C (ara Δ(lac-pro)13) as described (109) except that the DE-52 and Sephacryl S-1000 steps were replaced by Q Sepharose (Amersham-Pharmacia) FPLC eluted with a linear gradient of 200 - 550 mM ammonium chloride. This was followed by loading the fractions containing RecA onto a single-stranded DNA cellulose column (Sigma) equilibrated with 25 mM NaCl and eluting with a solution of 500 mM NaCl and 2 uM ATP. Analysis of the purified protein by SDS-PAGE showed no visible contaminants. The concentration of RecA was determined by the ninhydrin protein assay (128). MutS protein was purified as described previously (31) with minor modifications (10). MutSΔ680 protein was purified as described previously (21). MutL protein was a gift from Francisco J Lopez de Saro and M. ODonnell (Rockefeller University) and MutL and Ssb were obtained from USBiologies. PhiX174 RFI and virion DNA forms were
from New England Biolabs and the RFI form was digested with XhoI restriction endonuclease (New England Biolabs) to produce the linear duplex form.

Platination and survival of phiX174 DNA: PhiX174 DNA was platinated by reacting various cisplatin molar equivalents to 1 molar equivalent of ssDNA or RFI DNA in 5 mM sodium phosphate buffer, pH 7.4. The reaction was allowed to proceed for 16-24 hrs at 37°C, after which the DNA was precipitated with ethanol and resuspended in water. The average number of cisplatin adducts was determined by flameless atomic absorption spectroscopy. Aliquots of double-stranded platinated RFI and unmodified DNA were mixed with strains MV1161 (wild) and MV3855 (uvrA6), mixed with top agar and poured onto L broth plates solidified with 1.6% agar. The plates were incubated overnight at 37 °C before scoring the plaques.

Strand Exchange Assay: Reaction mixtures contained 25 mM Tris-acetate (pH 7.5), 10 mM MgOAc, 5% glycerol, 1 mM DTT, 8 mM phosphocreatine, 10 units/ml creatine kinase, 1 nM single stranded circular DNA and 6.7 μM RecA. Reaction mixtures were pre-incubated at 37°C for 10 min, linear duplex DNA substrate was added to a concentration of 0.8 nM and incubated an additional 10 min. Strand exchange was initiated by addition of a premixed solution containing 2 μM Ssb and 3 mM ATP. MutS and/or MutL were added one minute prior to initiation of the reaction or 30 min later. Samples were taken at indicated times and strand transfer was terminated by addition of 2 μl of buffer containing 5% SDS, 20% glycerol, 60 mM EDTA and Proteinase K to a
concentration of 1 mg/ml. After incubation at 42 °C for 30 minutes, samples were analyzed by electrophoresis in a 0.8% agarose gel with 40 mM Tris-acetate, 2 mM EDTA. Gels were processed by staining in Vistra Green (Amersham) fluorescent stain (1:10,000) for 60 minutes and then analyzed by Image Reader 1 Laser/l Image at 473nm on a Fuji FLA-5000 phosphorimager. The gels were quantitated using Image Gauge software v. 3.1.
Results

Survival of Strains Exposed to Cisplatin.

We have used deletion mutations in the dam, mutS and mutL genes to measure survival of strains containing them to cisplatin exposure. The data in Figure 3-1, show that the dam mutant is more sensitive to cisplatin than its isogenic mutS or mutL derivative. The dam mut strains are marginally, but consistently, more sensitive than wildtype. These results confirm our previous observation using presumed base substitution mutations that inactivation of MMR in a dam background indeed results in drug resistance (69).

Platination and Survival of phiX174 DNA.

Single-stranded phiX174 DNA molecules were reacted with various molar ratios of cisplatin to DNA and under the conditions of platination we have used, GG and AG intrastrand crosslinks constitute about 65% and 25% of the adducts respectively, and GNG intrastrand crosslinks about 5-10%. Interstrand crosslinks comprise about 2% of the total adducts in double-stranded DNA and there are trace amounts of monoadducts (6, 8). At the levels of platination we have used the contribution of interstrand crosslinks in single-stranded DNA should be minimal. The levels of adduction at the predicted ratios of 4 and 8 adducts per DNA molecule were measured by flameless atomic absorption spectroscopy and were in good agreement with the predicted values. In the same
Figure 3-1. **Survival of E. coli strains after exposure to cisplatin.**

Logarithmic-phase cells were treated for 60 min with the indicated concentration of cisplatin and plated for survival. Circles, wildtype; upside-down triangles, *mutS dam* deletion mutant; triangles, *mutL dam* deletion mutant; squares, *dam* mutant.

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Figure 3-2. **Survival of platinated phiX174 RFI in wildtype and NER-deficient E. coli.**

RFI molecules, with the indicated number of cisplatin adducts, were mixed with an excess of wildtype (squares) or *uvrA6* (circles) bacteria and the number of plaque-forming units determined.
experiment, we also platinated phiX174 RFI (covalently closed) DNA and the relationship between transfection efficiency of this platinated RFI DNA in wildtype and NER-deficient (uvrA6) bacteria is shown in Figure 3-2. There was a lower survival of the treated phage DNA in the NER-deficient strain which was expected given the known requirement for NER to remove cisplatin adducts (75, 91).

RecA Strand Transfer with Unmodified Substrates.

The strand transfer reaction is shown schematically in the top panel of Figure 3-3. Under the conditions we have used, the reaction is about 90% complete in 30 min as measured by the appearance of the nicked-circular (NC) product or the disappearance of duplex linear substrate (L) (Figure 3-3 bottom panel). This is preceded by the formation of slowly migrating intermediate (I) structures which persist throughout the reaction. In the absence of single-stranded substrate, no nicked-circle product was produced during 90 min incubation, indicating that there was no ligase contamination in the RecA preparation (data not shown). Inclusion of MutS in the reaction up to 250 nM with unmodified substrates, had no significant influence on the rate or yield of the reaction (Figure 3-5). At concentrations higher than 250 nM, a slight inhibition was noted presumably due to non-specific binding to the single-stranded DNA substrate and/or intermediates (data not shown).

The linear duplex substrate was derived from the RFI (covalently closed, CC) form by almost complete cleavage with XhoI. The small amount of CC remaining was
Figure 3-3. **RecA-mediated strand exchange reaction.**

Top panel: Schematic representation of the reaction with single-stranded (S) and linear (L) DNA substrates, intermediate structures (I) and the nicked-circular product (NC). Bottom panel: Time course of the RecA reaction in minutes with unmodified substrates. CC represents covalently-closed DNA which was used as a constant marker to quantitate substrates and products.
used as a standard to quantitate substrate and product bands since it has no influence on the reaction and its concentration remains constant.

**RecA Strand Transfer with Platinated Substrates.**

The effect of 0, 1, 10 and 24 cisplatin adducts in single-stranded substrate molecules (5386 nt) on RecA-mediated strand-transfer is shown in Figure 3-4. Compared to the unmodified substrate where the reaction is 90% complete in 30 min, even a single adduct per genome resulted in a reproducible reduction in reaction rate and at 90 min the yield is about 90% compared to the unmodified substrate. At 10 or 24 adducts per genome the yield was reduced to about 50% and less than 5% respectively. These experiments were carried out using platinated single-stranded DNA and unmodified linear duplex to reduce the effect of cisplatin interstrand cross-links. Reversing the modification of these two species gave essentially the same results with respect to reduced RecA strand transfer (data not shown).

**RecA Strand Transfer with Platinated Substrates and MutS.**

As described above, addition of MutS has no effect on the RecA-catalyzed strand transfer reaction with unmodified substrates. In contrast, an inhibition of strand exchange occurs in the presence of MutS if one of the substrate molecules contains cisplatin adducts. As shown in Figure 3-5, addition of 25, 125 and 250 nM MutS results in a
Figure 3-4. Effect of cisplatin adducts on RecA-mediated strand transfer to form nicked-circle product

Circles, unmodified single-stranded substrate; open squares, one adduct per molecule; triangles, ten adducts per molecule; filled squares, 24 adducts per molecule.

Figure 3-5. Inhibition of RecA-mediated strand transfer by MutS.

Single-stranded DNA molecules modified with one (A), four (B), eight (C) or ten (D) cisplatin adducts were used as substrates for RecA-catalyzed strand exchange. In each panel, the reaction with unmodified substrate is shown for comparison (filled circle) to the modified substrate (open squares) and for the latter with 25 nM (triangles), 125 nM (filled squares) and 250 nM (open circles) MutS. The effect of adding MutS up to, and including, 250 nM to unplatinated substrate is shown by the crosses.
concentration-dependent inhibition of strand transfer when the single-stranded substrate molecule is modified with 1, 4, 8 or 10 adducts. In these experiments, MutS was added at the beginning of the reaction. We have also added MutS 30 min after starting the reaction and this has does not have any effect on slowing further product formation after its addition compared to its absence (Figure 3-6). Upon measuring substrate uptake, it was found that a minimal amount of substrate is used when MutS is added at the 30 minute time point compared to the reactions containing no MutS. Therefore it is likely that MutS is acting at the joint molecule formation step of the reaction, binding to the platinated lesions, thus preventing recombination. At addition at 30 minutes, these intermediates are already going on to form product and thus, the yield of product from the reaction does not significantly change compared to the reactions where there was no MutS addition.

The population of molecules modified to four or eight adducts each is expected to contain 3% or 0.02%, respectively, unmodified genomes as calculated using the Poisson distribution. As the amount of product formation at these adduct concentrations exceeded these values, RecA can perform strand-exchange with adducted DNA but at a slower rate.

**MutS Δ680 does not Affect Strand Exchange.**

We have previously described a deletion mutation of *E. coli* MutS in which the terminal amino acid residues 680-853 are deleted from the protein and which confers a mutator phenotype on host cells indicating inactivation of the mismatch repair process (129). The mutant protein hydrolyzes ATP at the same rate as the wildtype protein but
Figure 3-6. Addition of MutS 30 Minutes After Initiation of the Reaction

Varying concentrations of MutS protein were added 30 minutes after initiating the reaction. (A) 1 adduct (B) 4 adducts (C) 8 adducts. The addition of MutS at any concentration once the reaction has started has very little affect on product yield.
has reduced ability to bind specifically to mismatched DNA, to form tetramers and
dimers and to interact with MutL (129). This mutant protein did not affect RecA-
mediated strand exchange at concentration up to and including 250 nM with either
modified or unmodified substrate (data not shown).

**Addition of MutL to the Reaction.**

We next added active MutL protein to the reaction expecting that there would be
enhanced inhibition of strand exchange in the presence of MutS. However, we have not
been able to show any specific inhibitory effect of MutL on the reaction except at high
MutL concentrations at which non-specific DNA binding occurs. We used two different
preparations of MutL each of which was active in an *in vitro* MMR repair assay. No
effect of MutL was found when various sub-optimal concentrations of MutS were used in
the reaction (data not shown).
Discussion

The mechanism by which MMR sensitizes human cells and E. coli dam mutants to the cytotoxic action of cisplatin is not known. The sensitization is undoubtedly related to the ability of MutS from E. coli and human cells to bind specifically to the platinated GG intrastrand crosslink (9, 74) but not to any other adduct including the interstrand crosslink (9). The affinity of MutS for the intrastrand crosslink is 10- to 40-fold less than that for a G-T mismatch for the human and E. coli enzyme, respectively (9, 74). It is possible that after exposure to cisplatin, MutS is bound to these adducts in the chromosome and is effectively titrated out leading to a temporary mismatch repair deficiency. If this interpretation were correct, however, we would expect to see drug resistance in a dam mutant at low doses or at short exposure times and this is not observed (Figure 3-1). As an alternative, we favor the idea that MutS binding to platinated DNA during RecA-mediated recombinational repair is the critical mode of action of MutS. We propose that inhibition of recombinational repair in dam mutants treated with cisplatin is central to MMR-mediated cytotoxicity. The results described in this paper suggest that it is both the reduction of RecA strand exchange activity by relatively few cisplatin adducts and the inhibition caused by the addition of MutS that blocks recombinational repair.
In *dam mutS* or *dam mutL* bacteria, there are few double-strand breaks (DSBs) due to MMR inactivation, the cells are not SOS stressed, and so there is adequate reserve recombinational capacity upon exposure to cisplatin. Although the MutS inhibition of RecA strand transfer is relieved in these strains due to MMR inactivation, the reduction in the rate of RecA activity with platinated substrate remains. With excess recombinational capacity, however, a reduction in rate may be inconsequential and the repair process simply takes longer to complete. This delay may favor removal of adducts by NER that otherwise would be substrates for recombinational repair. When a *dam* mutant, with limiting or no spare recombinational capacity, is overwhelmed with endogenous and cisplatin-induced DSBs, the recombinational repair process cannot be completed in the presence of MutS inhibition.

In wildtype strains there are only the cisplatin-induced DSBs to repair and, although MutS inhibition of RecA strand transfer should occur at the same frequency as in a *dam* strain, the cells are resistant to the action of cisplatin. This suggests that MMR inhibition of strand transfer is reversible and *in vitro* results using M13-fd heteroduplexes indicate that the RuvAB proteins are capable of this action (54). The reverse reaction produces the substrates and allows RecA to try again and, with the low affinity of MutS for the adduct, strand exchange will eventually occur. In *dam* mutants, however, we propose that the reverse reaction occurs infrequently, if at all, due to limited availability of RuvAB proteins (88) which are engaged in endogenous DSB repair.
It was proposed previously that recombination in *dam* mutants is performing near its maximal capacity to repair MMR-induced DSBs (88). Exposure of *dam* mutants to cisplatin would increase recombinational demand which could not be met resulting in unrepaired DSBs and subsequent cell death. This idea is supported by data showing that recombination is as critical as NER for survival in *E. coli* after cisplatin challenge (10). That MMR has an antirecombination function in homeologous recombination, and that MutS binds specifically to cisplatin lesions, suggested that this mechanism might be responsible for inhibiting recombinational repair (52, 56). In contrast to homeologous recombination, antirecombination action by MMR occurs on homologous DNA in which one of the recombining partner strands contains cisplatin adducts. This model is shown schematically in Figure 3-7 where after the replication complex encounters the adduct, replication fork progression is blocked followed by regression, where the newly synthesized complementary strands pair to form a duplex with a flush end (90). This end becomes a substrate for RecBCD digestion which after encountering a Chi sequence to activate its 5'-exonuclease activity and promote the release of the RecD subunit, RecA strand exchange can occur (90, 98). This strand exchange reaction can occur opposite a cisplatin lesion as shown in Figure 3-7 albeit at a slower rate. It is the heteroduplex region formed at this step to which MutS binds and decreases strand exchange activity even further. Although MutS must dissociate from this complex, given the relatively low affinity for the cisplatin intrastrand crosslink, inhibition occurs *in vitro* (Figure 3-5). In the absence of MutS, strand exchange can occur past the adduct on the complementary strand and the Holliday junction intermediates are recognized and cleaved by the
Figure 3-7. **Proposed model for recombinational tolerance of cisplatin adducts and its inhibition by MutS.**

The replication complex encounters a cisplatin adduct (filled symbol to which MutS may or may not, be bound) and stalls after uncoupling of leading and lagging strand replication followed by fork regression. The duplex end formed by the new strands is digested by RecBCD which, after activation at a Chi site, promotes RecA strand exchange and the Holliday junctions are acted upon by RuvABC. The replication complex is reloaded onto the fork by the PriA pathway and the adduct is effectively bypassed. MutS inhibits the process at the RecA strand exchange step. In this Figure, only a replication blocking lesion in the leading strand is shown although gap repair in the lagging strand would follow the same scheme and inhibition by MutS.
RuvABC complex. At this stage, the adduct has been effectively bypassed by the replication fork and the PriA-dependent pathway can reload the replication complex at the fork (99, 101). The adduct can now be removed by NER. Support for this model includes the high sensitivity of priA and ruvABC mutants to cisplatin as well as recA and recBC strains (10, 79, 91).

An alternative to the above is the futile cycle model originally developed to explain the role of MMR sensitization of cells to the cytotoxic effect of methylating agents (21). In this case the replication machinery stalls at a cisplatin adduct and the replicative polymerase is temporarily replaced by a translesion polymerase (130), followed by restoration of the replicative polymerase after bypassing the lesion. Replication across the adduct is error-prone resulting in the insertion of the incorrect base opposite the platinum adduct and subsequent binding of MutS to the base mismatch to initiate repair. Since no "good" base match exists for the adduct, the MMR system continually removes and replaces bases in a futile cycle (21, 131). The stalled fork is unable to progress and eventually disintegrates. Evidence supporting the involvement of translesion polymerases includes bypass of the AG cisplatin crosslink by DNA polymerase V (the umuDC product) (69) and that SOS-induction also promotes translesion synthesis across GG cisplatin adducts (132).

Both models can be incorporated into the SOS stress response that occurs after challenge with cisplatin (79, 117). The initial phase of the SOS response is the rapid
induction of the Uvr proteins to remove cisplatin lesions. However, NER has a 50-fold lower affinity for GG and AG intrastrand crosslinks than GNG intrastrand crosslinks, increasing the chance that the replication fork encounters one of the GG or AG lesions (76). The second phase of the SOS response is recombinational repair of stalled or collapsed forks at these lesions as described in the first model above (117). The last phase of the SOS response is translesion synthesis across the lesions as described in the futile cycle model. In this scenario, there are two points in the SOS response at which inhibition of repair by MMR can occur.

MutL is clearly implicated in vivo in conferring cisplatin-resistance to dam cells (Figure 3-1) and in preventing homeologous recombination between related bacteria in vivo as well as preventing RecA-mediated strand exchange between closely related M13-fd bacteriophage DNAs in vitro (52, 54, 56). In the M13-fd experiments, MutL alone is without effect but it potentiates the inhibition caused by MutS. MutS alone inhibited the reaction by about 50% and this increased to about 90% in the presence of MutL (56). One difference between the M13-fd and platinated DNA experiments described here is the level of adducts or base mismatches per genome; 4 or 8 cisplatin adducts versus 192 base mismatches in the M13-fd strand exchange reaction (133). The difference in results between mismatches and platinum adducts may reflect a dose-response relationship where at low levels of adducts or mismatches MutS does not need the potentiating effect of MutL for antirecombination and the inhibition of strand transfer is not as drastic. In this model, MutL is not required to cooperate with MutS to inhibit strand exchange in the
in vitro assay where the few cisplatin crosslinks are present, but MutL is required in vivo
where a larger number of adducts are predicted to be present (Figure3-1). The inhibitory
effect of a cisplatin adduct on RecA action is presumably due to the introduction of a 50°
bend toward the major groove and concomitant widening of the minor groove (11). In
contrast, many base mismatches induce little deformation of the DNA (126). We know of
no in vitro data where fewer mismatches have been used than the 192 in the M13-fd
strand exchange reactions. Alternatively, both the number of adducts or mismatches and
their distribution may be important for efficient inhibition by MutS. While the
mismatches are scattered throughout the genomes of M13 and fd heteroduplexes (133),
the distribution of cisplatin adducts in the phiX174 DNA is not known. Single-strand
annealing between homeologous substrates in budding yeast has been shown to be
reduced by the MutS homologues, Msh2 and Msh6, but not by the MutL homologue
Pms1, an outcome similar to the result we have described here (134).

It may be that the in vitro RecA-mediated strand exchange reaction does not
contain all the components to accurately reflect antirecombination in vivo and that an
additional factor(s) is required for interaction of MutL with MutS and platinated DNA. A
possible candidate protein is the DNA polymerase III holoenzyme beta clamp (PCNA is
the eukaryotic homolog) encoded by the dnaN gene. The beta clamp has been shown to
interact with MutS (135) and the holoenzyme is required for MMR re-synthesis after
exonucleolytic digestion to remove mismatches (126). We are explore if dnaN mutations
affect recombinational repair in Chapter V.
Given the conservation of the mechanism and proteins of MMR between *E. coli* and humans, it is possible that the same mechanism of cisplatin-induced cytotoxicity may also apply in human cells. We note that cisplatin has been most strikingly successful in the treatment of testicular tumors (3) which occur in a tissue undergoing obligatory meiotic recombination and in which abrogation of recombinational repair is expected to promote apoptosis.

Acknowledgements

We thank Mary Munson for her invaluable help and guidance in protein purification, Katie Barnes and Jennifer Robbins for instruction in flameless atomic absorption spectroscopy and Mike Volkert for the *E. coli* strains. This work was supported by grant GM63790 from the National Institutes of Health.
CHAPTER IV

SEPARATION OF MUTATION AVOIDANCE AND ANTIRECOMBINATION FUNCTIONS IN AN _ESCHERCHIA COLI MUTS_ MUTANT

Abstract

DNA mismatch repair in _Escherichia coli_ has been shown to be involved in two distinct processes: mutation avoidance, which removes potential mutations arising as replication errors, and antirecombination which prevents recombination between related, but not identical (homeologous), DNA sequences. We show that cells with the _mutSΔ800_ mutation on a multicopy plasmid are proficient for mutation avoidance but are defective for antirecombination. In interspecies genetic crosses, recipients with the _mutSΔ800_ mutation show increased recombination by up to 280-fold relative to _mutS^+_. The MMR status of an _E. coli dam_ mutant directly relates to its sensitivity to the cytotoxic agents cisplatin and MNNG. Like _mutS^+_ , cells with the _mutSΔ800_ mutation are sensitive to MNNG but unlike _mutS^+_, are resistant to cisplatin. The MutSΔ800 protein binds to a one base deletion/insertion loop and a G-T mismatch, but not to intrastrand platinated G-G crosslinks. The results indicate that the C-terminus of MutS, which is required for tetramerization of the protein, plays a critical role in the antirecombination function of MutS as well as in cisplatin sensitization, but less significant for mutation avoidance and MNNG sensitization.
Introduction

The Dam-directed mismatch repair (MMR) system of *Escherichia coli* removes potential mutations arising as replication errors (mutation avoidance) (126, 136, 137). Correction of biosynthetic errors by MMR occurs directly after replication, just behind the replication fork, where the parental DNA strand is fully methylated at GATC (*dam*) sequences and the newly-synthesized strand is not yet methylated. When base mispairs arise in such hemi-methylated DNA, they are bound initially by the MutS protein which subsequently recruits MutL and MutH to form a ternary complex. Incision by activated MutH occurs on the unmethylated strand at a nearby GATC sequence, followed by excision, in either the 3'- or 5'-direction, of the mismatched base pair and surrounding sequence. Re-synthesis by the replicative polymerase, DNA polymerase III, restores the correct nucleotide sequence and the resulting nick is sealed by DNA ligase to complete the repair process. Subsequently, the repaired DNA strand is methylated at GATC sequences by Dam methyltransferase and this methylation prevents further MMR action. This model has both genetic and biochemical support including a mutator phenotype associated with *mutS* mutants (126, 137).

MMR also plays a role in preventing recombination between related, but not identical (homeologous), DNA sequences (anti-recombination) (52). Genetic crosses
between *Escherichia coli* and *Salmonella enterica* Serovar Typhimurium are sterile unless the recipient contains mutations in the *mutS* or *mutL* MMR genes. This result suggests that MMR either impedes or actively reverses recombination intermediates or destroys them, with the former having some experimental support (54, 68, 138). On a biochemical level, MutS and MutL block RecA-mediated strand exchange between the fd and M13 genomes, which are 3% divergent, but not between M13-M13 genomes (56). In this reaction, MutS has a greater effect than MutL, which is effective only in combination with MutS. The intimate connection between mutation avoidance and antirecombination is shown by the phenotype of *E. coli mutS* mutations which are defective in both these processes. A survey of a large number of *E. coli mutS* mutants failed to detect any with only one of the phenotypes (139). Similarly, we have been unable to find a mutant with only one of these phenotypes among a collection of dominant-negative alleles (140).

*E. coli dam* mutants are more sensitive to the cytotoxic action of cisplatin and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) than wildtype (21, 69). Mutations in genes that incapacitate MMR (*mutS, mutL* or *mutH*) in a *dam* background confer a level of resistance to these agents similar to that of the wildtype. This result indicates that inappropriate MMR on chemically modified DNA can sensitize *dam* cells to these cytotoxic agents (21, 69, 141). MMR in wildtype cells is restricted to one strand in the hemimethylated region behind the replication fork because MutH endonuclease cannot use fully methylated DNA as a substrate. In *dam* mutants, however, MMR can occur on either strand anywhere on the chromosome because MutH can use either DNA strand as a substrate. It has been proposed that MMR-induced single-strand breaks or gaps can be
converted to double-strand breaks (DSBs) either by MutH action at the same GATC sequence on the opposite strand (45) or by replication fork collapse (88, 142). The DSBs so formed require recombination for their repair thereby making recombination essential for viability (88). As a result of these DSBs, recombination capacity is limiting (88) and when dam cells are exposed to cisplatin a large number of DSBs accumulate (142). MMR is required for the formation of cisplatin-induced DSBs (142) suggesting that they probably occur at cisplatin lesions undergoing MMR at either unreplicated lesions or those formed with mismatches after the action of translesion polymerases. The accumulation of DSBs in dam mutants, which have limited recombination capacity, and the reduced ability of MutS to prevent RecA-mediated strand transfer of platinated DNA during recombinational repair (33) provides a plausible model to explain why MMR sensitizes dam cells to cisplatin.

To explain MMR sensitization of E. coli dam mutants by MNNG, a model incorporating "futile cycling" can be proposed (21). In this model, the replicative polymerase inserts either a T or C opposite template O6-methylguanine (O6-meG). Since neither of these bases is considered a "good" match by the MMR system, a futile cycle of insertion and removal ensues preventing progression of the replication fork. The blocked fork may eventually disintegrate requiring recombinational repair to restore it. Futile cycling can also occur at sites away from the replication fork as a consequence of MMR recognition and MutH incision on unmethylated DNA at O6-meG-C base pairs to promote DSB formation (141). It is the latter that occurs in dam mutants, but not in wildtype, and may be principally responsible for MMR sensitization to MNNG.
The MutS protein binds specifically to heteroduplex DNA containing base mismatches (31) or small insertion/deletion loops (IDLs) (24) as well as platinated G-G intrastrand crosslinks (9) and O\(^6\)-meG mispaired with either C or T (19). The crystal structure of *E. coli* MutS bound to an oligonucleotide with a G-T mismatch has been solved using a derivative of the MutS protein, MutS\(\Delta 800\), which lacks the C-terminal 53 amino acids (143). The MutS\(\Delta 800\) mutant crystallizes as a dimer and retains the ability to bind DNA and ATP, just as full length MutS does (143). Further analysis into the properties of the MutS and MutS\(\Delta 800\) proteins by equilibrium sedimentation and gel filtration show that MutS dimers can assemble into higher order oligomeric structures, while the MutS\(\Delta 800\) mutant is restricted to dimer formation only (32). The ability of MutS to form tetramers suggests this oligomeric state is important in MutS function. A similar conclusion was reached with the MutS protein from *Thermus* species (144, 145) for which a crystal structure is also available (146). Here, we show that the C-terminal end of MutS is critical for antirecombination and cisplatin sensitization but less significant for mutation avoidance.
Materials and Methods

Strains, plasmids and media: Strains GM3819 (Δdam-16::Kan), GM4799 (mutS458::mTnl10Kan), GM5550 (recA56 mutS458::mTnl10Kan), and GM5556 (Δdam-16::Kan mutS215::Tnl0) are derivatives of AB1157 (thr-1 ara-14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 supE44 galK2 rfbD1 mgl-51 rpsL31 kgdK51 xyl-5 mtl-1 argE3 thi-1). AB259 (HfrH) was obtained from E.A. Adelberg (Yale University). Salmonella strains SA536 and SA977 were obtained from the Salmonella Genetic Stock Centre, University of Calgary, Canada. Plasmids with the mutSΔC800 (143) (referred to as mutSΔ800 in this paper) and mutSΔ680 (129) were derived from pMQ372 (129) carrying the mutS+ gene. Full descriptions of strains and plasmids can be found at http://users.umassmed.edu/martin.marinus/dstrains.html. Bacteriological media have been described previously (88, 147) except for the MacConkey Agar Base (Difco) medium which was supplemented with 50 g/l galactose. Ampicillin, rifampicin, and streptomycin were included in media, when required, at 100 µg/ml.

Estimation of mutant frequency, conjugational crosses and cytotoxicity: Spontaneous mutant frequencies were measured as described elsewhere (80, 147) and conjugation experiments were performed as described previously (88) except that mating mixtures were incubated for 1 hour at 37°C for homologous crosses and 3 hours for the homeologous crosses. Cell survival after exposure to cisplatin or MNNG was measured as described previously (21, 80). Each experiment was performed at least twice and proved to be reproducible, therefore only the results of a single experiment are shown.
Proteins and DNA: MutS protein was purchased from USB and dialyzed before use against 20 mM KPO4, pH 7.4, 0.1 mM EDTA, 1 mM PMSF and 10 mM beta-mercaptoethanol. We found that heteroduplex binding (see below) and ATPase specific activity (data not shown) were the same as that for the MutSΔ800 protein. MutL protein was a gift from F. Lopez de Saro and M. O'Donnell (Rockefeller University). MutSΔ800 was purified from strain GM7854 (pmutSΔ800/BL21(lambdaDE3)). Briefly, the cells were grown at 37°C to an OD600 of 0.8 and shifted to 26°C for induction with 1mM IPTG. The cells were grown an additional 3 hrs at 26°C before harvesting. The cells were lysed using a Microfluidizer (Microfluidics Corp.) and then centrifuged at 15K for 30 minutes. The supernatant was treated with streptomycin sulfate and ammonium sulfate as described previously (31). The resulting fraction was loaded onto a heparin agarose column (Amersham Pharmacia) and eluted with a linear gradient of 100-400 mM KCl. The fractions containing MutS were subsequently loaded onto a ceramic hydroxylapatite column (Sigma) and eluted using a linear concentration gradient of 20 mM-120 mM KCl. This produced a single MutS peak eluting at 50-70 mM KCl. Fractions containing MutSΔ800 were pooled, concentrated using a Centriprep column (Millipore) and frozen at -75°C. The protein was at least 95% pure as determined by SDS-PAGE analysis. Protein concentration was determined by ninhydrin analysis (128).

M13mp18 DNA RFI and single-stranded circular DNA forms were from New England Biolabs (NEB). The RFI form was digested with DraIII and BglIII restriction endonucleases (NEB) to remove lac operon DNA. Fd single-stranded circular DNA was
prepared by growing a culture of strain AB259 to about $1 \times 10^8$ cells, adding fd at a multiplicity of 1 and allowing the culture to grow for an additional 3-4 hours. The culture was centrifuged to remove bacteria and 20% polyethyleneglycol 8000-2.5M NaCl solution was added to the supernatant (1:3) and left on ice for 30 minutes. The mixture was centrifuged at 9K for 10 min and the phage pellet resuspended in TE buffer. The DNA was phenol extracted, ethanol precipitated, and its concentration determined by UV spectroscopy.

**Heteroduplex construction, electrophoretic mobility shift assay and RecA strand exchange assay:** Heteroduplex DNA with a single IDL (Insertion/Deletion Loop) and labeled with P$^{32}$ was constructed and analyzed by electrophoretic mobility shift assay as described previously (24, 129). A DNA oligonucleotide provided by Jennifer Robbins and J.M Essigman (MIT), designated Xlink (5'- CCT CTC CTT GGT CTT CTC CTC TCC- 3') contains a cisplatin crosslink between the two guanines and was annealed to its complementary sequence, or the complementary sequence to form two GG-TT mismatches. The platinated oligonucleotides were not radiolabelled. The P$^{32}$-labelled DNA and the platinated DNA, which was stained with Vistra Green (Amersham-Pharmacia) for 1hr, were quantitated using a Fuji Phosphorimager. RecA-mediated strand transfer was measured as described except that M13 and fd molecules were used(33).
Results

Spontaneous mutation frequency of wildtype and mutS strains.

Strain GM4799, a mutS null mutant (129), was transformed with plasmids bearing the mutS*, mutSΔ680 and mutSΔ800 genes. The mutSΔ680 and mutSΔ800 designations indicate where the 853 amino-acid MutS protein was truncated. The mutSΔ680 mutation has a null mutator phenotype and the MutSΔ680 protein is unable to form oligomers and has reduced ability to interact with MutL (129). The transformed strains were tested for reversion of the argE3 and galK2 markers of the host strain as well as resistance to rifampicin. The results in Table 4-1 and Figure 4-1 show that the GM4799 strain with the mutSΔ800 or mutS* plasmids did not display a mutator phenotype but that GM4799 with mutSΔ680 plasmid did. The result with rifampicin resistance confirmed that obtained previously by Lamers et al (143) and Biswas et al (145). The reversion to Arg* and Gal* papillae formation was marginally higher in the GM4799 with the mutSΔ800 plasmid culture compared to mutS* but both these values were much lower than in the mutSΔ680 plasmid control strain. We conclude that the mutSΔ800 allele does not confer a mutator phenotype to the cell.
Figure 4-1. **Gal reversion assay.**

Red Gal\textsuperscript{+} revertants are shown on a background of white Gal\textsuperscript{-} colonies. From left to right the strains contained plasmids with wildtype \textit{mutS}, \textit{mutS}A\textsubscript{680-850} and \textit{mutS}A\textsubscript{800}.

<table>
<thead>
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<th>Plasmid allele</th>
<th>Arg\textsuperscript{R}</th>
<th>Rif\textsuperscript{R}</th>
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</tr>
<tr>
<td>\textit{mutS}A\textsubscript{800}</td>
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</table>

Cultures of strain GM4799 with the indicated plasmid allele were plated on media selective for rifampicin-resistance or arginine prototrophy. The numbers represent mutant colonies per $10^8$ cells plated.
Interspecies crosses.

Strain GM4799 with the mutS plasmids, was used as a recipient in conjugal crosses with *E. coli* (homologous) or *Salmonella enterica* Serovar Typhimurium (homeologous) donors. With the *E. coli* donor, AB259, recombinants were formed after 60 min of mating, at the same frequency with each GM4799 strain indicating no effect of the mutS alleles on homologous recombination (Table 4-2). With the *Salmonella* donors SA536 or SA977, however, the GM4799 recipient with the mutSΔ800 plasmid formed recombinants 283- and 100-fold higher than the wildtype recipient respectively. These values, however, are about half that for the GM4799 strain with the mutSΔ680 plasmid indicating that the mutSΔ800 allele does not completely relieve the inhibition produced by a fully proficient MMR system. The level of homeologous recombination was the same with either strain GM4799 or GM4799 containing the mutSΔ680 plasmid (data not shown).

To ensure that the results of the crosses described above were due to recombination, we repeated the crosses with a recombination-deficient (recA56) derivative of GM4799, GM5550. With the *E. coli* donor, AB259, no recombinants were detected in any of the crosses with the GM5550 recipients (data not shown). With the *Salmonella* donors, there was more than a 99% decrease in recombinant formation in the GM5550 strains with the mutSΔ800 and mutSΔ680 plasmids. We conclude that the mutSΔ800 mutation significantly reduces antirecombination in interspecies crosses in a recA-dependent manner.
Table 4-2. Yield of recombinants in homologous and homeologous crosses.

<table>
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<tr>
<th>Donor</th>
<th>Plasmid in recipient</th>
<th>Recipient</th>
<th>Selected Marker(s)</th>
<th>Frequency</th>
<th>Fold Increase</th>
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<td>Rec&lt;sup&gt;+&lt;/sup&gt;</td>
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</tr>
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</tbody>
</table>

Donor and recipient (GM4799, GM5550) cultures (at 1-2 x 10<sup>8</sup> cells per ml) were mated as described in Methods. The numbers in the Table represent the Thr<sup>+</sup> Leu<sup>+</sup> [Str<sup>+</sup>] or Arg<sup>+</sup> [Str<sup>R</sup>] recombinants in 50 µl of undiluted mating mixture.
Sensitivity to cytotoxic agents.

*E. coli* dam mutants are more sensitive to cisplatin and MNNG than wildtype (21, 69). Fig. 2A shows that a *dam mutS* strain, GM5556, carrying the *mutS*<sup>+</sup> plasmid is more sensitive to cisplatin than the same strain bearing the *mutSΔ680* (null mutation) plasmid or the *mutSΔ800* plasmid, indicating that the *mutSΔ800* gene product is unable to promote MMR sensitization. Figure 4-2A also shows that the survival of GM3819, a *dam mutS*<sup>+</sup> strain, with the *mutSΔ800* plasmid is the same as with a *mutS*<sup>+</sup> plasmid. This result indicates that the *mutSΔ800* allele in multicopy does not have a dominant-negative phenotype in wildtype (*mutS*<sup>+</sup>) cells.

MutS and MutSΔ800 binding to platinated oligonucleotides.

To investigate if MutS and MutSΔ800 have altered binding to cisplatin crosslinks, we tested the ability of both proteins to bind to homoduplex, heteroduplex and platinated DNA substrates in an electrophoretic mobility shift assay. The data in Figure 4-3 show that MutSΔ800 has approximately the same affinity for P<sup>32</sup>-labelled heteroduplex DNA with a single base IDL (Insertion/Deletion Loop) than MutS. There was no significant binding of these proteins to a fully base-paired homoduplex substrate under the same experimental conditions (data not shown).

The substrates for MutS binding to platinated DNA included unplatinated homoduplex DNA (negative control), unplatinated heteroduplex DNA with two G-T mismatches (positive control), and derivatives of each of these with a single intrastrand diguanyl cross-link (Figure 4-4). The mobility shifts shown in Figure 4-4, were
Figure 4-2. Survival of cells exposed to cisplatin and MNNG.

Survival after exposure to cisplatin (A) and MNNG (B) of wildtype mutS (filled circles), mutSΔ680-850 (inverted triangles), and mutSΔ800 (filled squares) plasmid-containing strains in the GM5556 (Δadam-16::Kan mutS::Tn10) strain background. The open squares in (A) represent the survival of mutSΔ800 in GM3819 (Δadam-16::Kan).
Figure 4-3. **Binding isotherm of MutS and MutSA800 to heteroduplex DNA.**

The top of the figure shows the binding of MutS and MutSA800 to a $^{32}$P-labelled heteroduplex DNA with a single IDL. The data are shown in the bottom part of the figure as a binding isotherm.
monitored by fluorescence after staining with VistraGreen. There were no detectable bands formed with MutS and MutSΔ800 to unplatinated homoduplex DNA (Figure 4-4A, lanes b-f). In contrast to the results in Figure 4-3, however, we did detect a difference in binding between MutS and MutSΔ800 to the unplatinated heteroduplex containing two adjacent G-T mismatches (Figure 4-4, lanes g-j). The addition of 4 pmols of MutS produced a discernable band shift (lane h) while, at the same concentration, MutSΔ800 did not (lane j). At this concentration, MutS forms two distinct bands (lane h), only one of which is present with MutSΔ800 (lane j) and may indicate that MutS first binds as a dimer and subsequently is converted to a tetramer. With 8 nM, the extent of heteroduplex binding by MutSΔ800 was about 80% that of MutS (lanes g and i). The difference in the binding affinities of MutSΔ800 for the heteroduplexes used in Figure 4-3 and 4-4A could be due to different sequence contexts or the number of mismatches or different affinities for base mismatches versus a one base IDL. An additional alternative is that multiple MutS proteins array on the heteroduplex with two G-T mismatches but MutSΔ800 fails to do so. There was a significant difference in binding of MutS and MutSΔ800 to DNA with a GG crosslink opposite two C residues (Figure 4-4B, lanes a-e). In fact, no binding of MutSΔ800 to this DNA was observed (Figure 4-4B, lanes d and e) although MutS, at the same concentration, did produce a considerable band shift (Figure 4-4B, lanes b and c). On the other hand, the control DNA with a GG crosslink opposite two T residues was bound by MutSΔ800 to about the same extent as MutS at 4 nM but less so at 8 nM due to partial shifting (Figure 4-4, lanes f-j).
Figure 4-4. Binding of MutS and MutSA800 to Platinated DNA.

A. The binding of MutS and MutSA800 to unplatinated homoduplex (lanes a-e) and heteroduplex (lanes f-j) DNA. No enzyme, (lanes a, f); MutS, 8 pmol, (lanes b, g), 4 pmol (lanes c, h); MutSA800, 8 pmol (lanes d, i), 4 pmol (lanes e, j). The DNA was visualized by staining the gel with Vistra Green. B. The binding of MutS and MutSA800 to simple (lanes a-e) and compound (lanes f-j) cross-linked platinated DNA. Lane assignments are the same as in A.
Figure 4-5. **RecA-catalyzed strand transfer.**

The top of the figure shows a schematic of the reaction. Single-strand (SS) circular DNA reacts with linear (L) duplex to form intermediate (I) structures which are converted to nicked-circle (NC) products. The fluorograph shows the results from the homologous M13-M13 (a-e) and the homeologous M13-fd (f-j) reactions. Samples were removed at time 0 (a, f), 5 min (b, g), 15 min (c, h), 45 min (d, i) and 90 min (e, j).
Figure 4-6. **Kinetics of MutS inhibition of RecA-mediated strand exchange.**

The graphs show the effect of varying concentrations of MutS (A.) and MutSΔ800 (B.) on RecA strand exchange using homologous (M13-M13) and homeologous (M13-fd). Filled circles, M13-M13; crosses, M13-M13 plus 100 nM protein; filled squares, M13-fd; open squares, M13-fd plus 25 nM protein; open circles, M13-fd plus 100 nM protein; triangles, M13-fd plus 25 nM protein plus 40 nM MutL.
Worth et al (56) showed that MutL stimulates MutS binding to M13-fd homeologous duplexes when the latter is at a sub-optimal concentration. In the experiment shown in Figure 4-6, 25 nM MutS or MutSΔ800 only partially inhibits RecA-catalyzed strand transfer. Inclusion of MutL, at 15 or 40 nM to these reactions, however, completely abolishes RecA transfer activity (Figure 4-6) indicating that MutL dimers can interact with MutS or MutSΔ800. MutL by itself had no effect on the rate or product yield in the reaction.

**Discussion**

The results described in this report show that MutS and MutSΔ800 proteins, produced from multicopy plasmids, impart different properties to cells. Although cells with the MutSΔ800 or MutS protein are not mutators (Table 4-1, Figure 4-1), the MutSΔ800-containing cells are deficient in antirecombination (Table 4-2) and not sensitized to cisplatin cytotoxicity (Figure 4-2A). This is the first demonstration of a separation of function phenotype for an *E. coli*mutS mutation. We propose that the C-terminal domain of MutS is required for efficient antirecombination and cisplatin sensitization, but not mutation avoidance or MNNG sensitization. Given that MutSΔ800 can only form monomers and dimers, we postulate that the inability to form tetrameric MutS could be responsible for this phenotype.
The results we have obtained in this report, postulating the requirement of MutS tetramer formation, are best explained by the findings of Bjornson et al (32) who have argued that the MutS tetramer is likely the active form of MutS in mutation avoidance. To support this conclusion, they found that MutSΔ800 protein bound to mismatched DNA less efficiently than MutS. Our results also indicate that MutSΔ800 does have reduced affinity for GG-TT base mismatches (Figure 4-4) but not with the one base IDL (Figure 4-3). Given that MutSΔ800 has reduced affinity for base mismatches, we propose that the lack of a mutator phenotype in cells over-expressing MutSΔ800 (Table 4-1, Figure 4-1) can be explained by the MutS dimer being able to cope with the few mismatches generated behind the replication fork. In interspecies crosses, however, the much larger number of mismatches overwhelms the MutSΔ800 protein's reduced mismatch binding ability, thereby leading to reduced antirecombination as shown in Table 4-2. A crucial part of our proposal, therefore, is that the cell's response when limited to using only dimeric MutS depends on the number of mismatches involved; if there are few, overproduced dimeric MutS can substitute for the need for wildtype MutS at its normal cellular concentration. The data in Figure 4-6 showing a greater amount of M13-fd heteroduplex formation by MutSΔ800 compared to MutS, is consistent with this model. The combination of MutL and MutSΔ800 is as effective as MutS and MutL where the level of mismatching is 3%. We predict that at higher levels of mismatching, such as the 17% in *E. coli - Salmonella* crosses, MutSΔ800 would be much less effective than MutS, even in the presence of MutL.
Bjornson et al (32) also showed that MutH-induced incision of heteroduplex DNA, in the presence of MutL, was reduced by MutSΔ800. This observation can also help to explain the response of dam mutants with the over-expressed MutSΔ800 protein to cisplatin and MNNG. For cells exposed to MNNG, there is sufficient binding of MutSΔ800 to O⁶-meG mismatches to provoke a sensitization response even though there is reduced MutH-induced incision (Figure 4-2B). For dam cells expressing MutSΔ800 and exposed to cisplatin, however, the reduced binding of MutSΔ800 to intrastrand diguanyl-cisplatin crosslinks and the reduced MutH-induced incision activity allow the cell to repair, by recombination, the few DSBs that might be formed (Figure 4-2B).

The model proposed above regarding the number of mismatched base pairs and the ability of over-expressed MutSΔ800 to deal with them makes the strong prediction that when MutSΔ800 is expressed from a single-copy gene, the dam cells containing it would have different responses for spontaneous mutagenesis and resistance to cisplatin and MNNG compared to expression from a multicopy plasmid. These experiments are in progress.

Surprisingly in Figure 4-2, MutSΔ800 when expressed in a wildtype background does not display a dominant negative phenotype in regards to cisplatin sensitivity. This result appears to rule out the possibility that the wildtype MutS, which is at a low concentration, could be titrated out by the plasmid expressed MutSΔ800, leading to a
dominant negative phenotype. We have not yet quantitated the protein levels of the plasmid expressed MutSΔ800 versus the chromosomally expressed copy of MutS. One possibility to explain the lack of dominance is that a mixed population of oligomers containing MutS and Δ800 is being produced, allowing for near-full functionality of the protein, which is absent in a population containing only the MutSΔ800 (Figure 4-2 and 4-4B). As evident from the equivalent levels of survival from the experiment exposing the cells to MNNG, a dimeric version of MutS containing a wildtype and MutSΔ800 monomer would likely be sufficient for mutation avoidance functions. This is also supported by similar binding affinities of MutS and MutSΔ800 for the IDL and the G-T mismatch. With regard to cisplatin, if a mixed dimer population is capable of interaction with one another forming a pseudo-tetramer or tetramer like structure, this would offer another alternative for the lack of dominance upon expression of MutSΔ800 in the wildtype background. As a result, the survival upon cisplatin exposure would be like that of wildtype. Important to note is that this hypothesis is reliant on a requirement for tetramerization and this mixed population phenomenon is not possible in the in vitro experiments.

The results in Figure 4-4, indicate that the C-terminal end of MutS is required for binding to platinated GG-CC crosslinks which could also indicate a requirement for MutS tetramerization, in that the dimeric Δ800 cannot recognize the lesion, whereas the wildtype which can form tetramers does. At present, it is not known how this crosslink opposite CC differs in structure from that opposite TT to require the wildtype form of
MutS. How MutS distinguishes G-T and G-C base pairs, as well as the platinated GG crosslink remains unclear, but the work presented here offers some interesting insights. It is possible that binding to a platinated lesion requires critical contacts with residues residing in the C-terminus of MutS, offering another alternative to the tetramerization requirement. Furthermore, the deletion of the C-terminus may disrupt the binding of MutS to lesions in general, where the ability to bind a lesion for which MutS has a lower affinity, such as the intrastrand GG crosslinks (10-40 fold lower than a G-T mismatch), is now significantly diminished. Either explanation is supported by the survival results for MutSA800 exposed to cisplatin (Figure 4-2). If this is plausible, this would infer that recognition of mismatches by MutS is different than recognition of a platinum lesion. As there is no crystal structure of MutS bound to a platinum lesion, nor is there a structure of MutS containing its C-terminal end bound to any lesion, it is difficult to determine whether this is the case. In addition to these alternatives to tetramerization, we do not rule out a critical loss of interaction(s) between the mutant MutSA800 and other proteins, which can ultimately affect the efficiency of recognition or repair processes. To clarify, these interactions could be affected by the loss of the C-terminal end in general, or a requirement of the protein to interact with a tetrameric MutS, neither of which is met in the mutSA800 mutant.

That MutS can bind to platinated GG-CC crosslinks suggests that MMR-induced DSBs can occur in unreplicated DNA of dam mutants at these sequences, as well as crosslinks opposite T bases, which can be formed by translesion polymerases. Platinated
GG-CC crosslinks can also be formed during recombinational repair of DSBs and these are bound by MutS which can block further branch migration (33), thereby preventing DSB repair. The ability of MutSΔ800 to perform an antirecombination function with the platinated lesions as shown in Figure 4-2 is severely abolished, and we propose this is due to any combination of the reasons described above.

Finally, we note that mammalian cell lines also show sensitivity to cisplatin and MNNG and MMR-deficient lines derived from them are resistant to both compounds (73, 131, 148), although whether cisplatin resistance is due specifically to MMR-deficiency has recently been challenged (62, 149). Cisplatin-resistant cells isolated from patients treated with this drug have also been shown to be deficient in MMR (67). Like the bacterial MutS protein, the human MutS-alpha counterpart also binds to cisplatin intrastrand crosslinks and O6-meG mismatches (74). It will be interesting to find out if the models and ideas we have formulated with E. coli in this and previous papers (10, 33, 80, 142) can be extended to mammalian cells.

Acknowledgements

We thank Meindert Lamers and Titia Sixma for the mutSΔC800 plasmid; Kenneth Sanderson for the Salmonella donor strains; Jennifer Robbins for construction and purification of the cross-linked platinated oligonucleotides; Dianne Schwarz for assistance with the radiolabeled band shifts; F. Lopez de Saro and M. O'Donnell for the
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purification. This work was supported by grant GM63790 from the National Institutes of
Health.
CHAPTER V

DIFFERENTIAL EFFECTS OF CISPLATIN AND MNNG ON DNA MUTANTS OF
ESCHERICHIA COLI

Abstract

DNA mismatch repair (MMR) in mammalian cells or Escherichia coli dam mutants increases the cytotoxic effects of cisplatin and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). We found that dna mutants, and their dam derivatives, at the permissive temperature for growth, respond differently to the two agents. Most striking are the results for the dnaE46 (alpha catalytic subunit) mutant, and a DnaX over-producer, which are sensitive to cisplatin but resistant to MNNG. On the basis of these results we propose that the lesions produced by these two agents can affect chromosome replication in two ways. They can block progression of replication forks which then disintegrate or collapse and they promote fork collapse during MMR at lesions in unreplicated DNA. In both cases recombinational repair is required to restore active replication forks and this process can be inhibited by MMR. We also found an elevated spontaneous mutation frequency to rifampicin resistance in dnaE486 (10-fold), dnaN159 (35-fold) and dnaX36 (10-fold) strains. The mutation spectrum in the dnaN159 strain is not consistent with a loss of interaction between MutS and the product of the dnaN gene, the beta-clamp of the polymerase III holoenzyme.
Introduction

Cisplatin (cis-diaminodichloroplatinum (II)) is an antitumor agent which has a cure rate of greater than 90% for testicular cancer (3). It reacts with DNA to produce mostly intrastrand crosslinks between adjacent guanines (65% of the total), adjacent guanine and adenines (25%), and guanines separated by a base (1,3-GNG, 5-10%) (7, 8). Interstrand crosslinks comprise about 2% of the total adducts and small amounts of monoadducts are also formed. The biologically inactive trans isomer of cisplatin also produces 1,3-GNG adducts, interstrand crosslinks and monoadducts (6, 15), suggesting that intrastrand crosslinks between adjacent purines are the biologically important adducts of cisplatin since they efficiently block progression of DNA polymerases in vitro and in vivo (14).

Nucleotide excision repair (NER) removes platinated intrastrand crosslinks but the 1,3-GNG lesions are removed at a rate 50-fold faster than those between adjacent purines (76). The importance of NER is manifested by the increased sensitivity of NER-deficient mutants of Escherichia coli and mammalian cells to cisplatin (4, 91, 92). In addition, however, recombinational repair mechanisms are as important as NER in allowing cells to survive cisplatin damage since E. coli strains in which recombination is defective show increased susceptibility to cisplatin (10, 79). Cisplatin is also a potent inducer of recombination (80).
Methylating agents are used in cancer chemotherapy and laboratory versions, such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), react with DNA and cause a signature type of damage, producing a variety of lesions which include O^6^-methylguanine (O^6^-meG), 3-methyladenine and 7-methylguanine (18). These lesions are usually removed either directly, in the first case by a methyltransferase, or through base excision repair using specific glycosylases. O^6^-MeG-cytosine or thymine-containing base pairs are substrates for mismatch repair (MMR) through recognition by MutS (19) and the latter can be formed by replicative or bypass polymerase action (20).

GATC sequences in E. coli DNA are methylated directly after replication at the N6 position of adenine by Dam methyltransferase a product of the dam gene (30). The DNA behind the replication fork is already methylated on the parental strand but not on the newly-synthesized daughter strand to give hemimethylated DNA. Replication errors are present in the unmethylated strand and MMR is initiated when MutS binds to base mismatches (126). Recruitment of MutL and MutH forms a ternary complex which activates the latent endonuclease activity of MutH to cleave 5’ to the G at an unmethylated GATC sequence. The UvrD helicase unwinds the unmethylated strand in either the 3’ or 5’ direction and which is digested by exonucleases with a distinct polarity. Re-synthesis is accomplished by the polymerase III holoenzyme complex and the resulting nick is ligated by DNA ligase (126). In wildtype cells, mismatch correction is restricted to the hemimethylated region as MutH has little, if any, activity on fully methylated DNA (35). In dam mutants, which lack DNA adenine methyltransferase, no
methylation is present and MMR can occur in newly replicated as well as in unreplicated DNA because MutH can utilize unmethylated DNA as a substrate (88).

Mammalian cells are sensitive to the cytotoxic action of MNNG (93) and cisplatin (67) but MMR deficient cell lines derived from them are resistant to the action of these drugs, although this association for cisplatin has recently been questioned (62, 149). *E. coli dam* mutants are also more sensitive to the cytotoxic effects of cisplatin (69) and MNNG (21) than wildtype. Mutations disabling the MMR system (*mutS, mutL*) in a *dam* cell, however, render it as resistant to these agents as wildtype (21, 69). The MutS protein from *E. coli* and human cells specifically recognizes the platinated GG intrastrand crosslink (9, 74) and O^6^-meG-cytosine and thymine base pairs (9, 74). Although the mechanism by which MMR sensitizes cells to MNNG and cisplatin is not known, it has recently been shown that MutS reduces RecA-mediated strand exchange using a platinated DNA substrate, suggesting that MMR interferes with recombinational repair of lesions (33).

Although MMR sensitizes cells to both cisplatin and MNNG cytotoxicity, it is unlikely that the identical mechanism(s) applies for each agent. Platinated intrastrand crosslinks are replication blocking lesions *in vitro* and *in vivo* (14) but O^6^-meG in template DNA can be replicated *in vitro* and *in vivo*, although a high mutation frequency results from mispairing with thymine (18). The O^6^-meG-cytosine and -thymine base pairs formed during chromosome replication are both substrates for MMR and the futile
cycling between them was proposed as a mechanism to explain sensitization (21). Such a mechanism cannot apply to platinated adducts in template DNA during chromosome replication, although it could apply when translesion polymerases replace replicative polymerases (130). If, indeed, the replicative polymerase can use O\(^6\)-meG as a template base but is blocked by cisplatin adducts, then strains mutant for polymerase III holoenzyme and associated proteins might show a differential sensitivity to MNNG versus cisplatin. We report here results consistent with this hypothesis using \textit{dnaB} (helicase), \textit{dnaE} (catalytic subunit), \textit{dnaG} (primase), \textit{dnaN} (beta-clamp) and \textit{dnaX} (tau and gamma subunits, with the latter being part of the clamp loader complex) mutants.

We recently described a mutated MutS protein, MutS800, produced from a multicopy plasmid, that sensitizes cells to MNNG but not to cisplatin, suggesting different modes of MMR sensitization (142). In this report, we have extended this differential sensitivity of these two agents to \textit{dna} mutants. In addition, the MutS protein has been reported to interact with the beta-clamp (135) which confers processivity on the holoenzyme by "clamping" the catalytic subunit, DnaE, to DNA. If the interaction between the beta clamp, a product of the \textit{dnaN} gene, and MutS is diminished in the \textit{dnaN159} mutant, this might lead to a mutator phenotype. We show that the \textit{dnaN159} mutant does indeed have a mutator phenotype but it is not due to lack of MMR.
Materials and methods

Bacterial strains and plasmids: The bacterial strains used in this study are listed in Table 5-1. J. R. Walker's laboratory (150) constructed and designated strain GM36 (dnaX36), but it is called GM8051 in this paper to avoid confusion with the strain of the same name but different genotype derived in this laboratory. The dnaX plasmid, obtained from J.R. Walker (University of Texas, Austin), is a derivative of pBR322 (151).

Media: L medium contains 20 g tryptone (Difco), 10 g yeast extract (Difco), 0.5 g NaCl, 4 ml of 1M NaOH per liter and solidified when required with 16 g of agar. Brain Heart Infusion (Difco) broth was prepared using 20 g of powder per liter of water and solidified, when required, with 16 g of agar. Minimal medium was prepared as described by Davis and Mingioli (111) and supplemented with amino acids (80 μg/ml) as required. Ampicillin and rifampicin were included in media at 100 μg/ml while, tetracycline and chloramphenicol were included in media at 10 μg/ml. Kanamycin was included at 20 μg/ml when required. Media for the reversion assays contained 10% lactose and Xgal (40 ng/ml) in minimal media.

Estimation of rifampicin-resistant mutant frequency: Multiple cultures (generally 10) of the deletion strains and their isogenic partners were generated from an inoculum of a few hundred cells and grown to saturation. Portions of the cultures were plated on BH
Table 5-1. *E. coli* K-12 strains used in this study.

<table>
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<th>Number mutation/strain</th>
<th>Description</th>
<th>Source of</th>
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<td>E.A Adelberg</td>
</tr>
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<td></td>
<td>tsx-33 supE44(AS) galK2(Oc) hisG4(Oc) rfbD1</td>
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<tr>
<td></td>
<td>mgl-51 rpoS396(Am) rpsL31(StrSR) kgdK51 xylA5</td>
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</tr>
<tr>
<td></td>
<td>mtl-l argE3(Oc) thi-l</td>
<td></td>
</tr>
<tr>
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<td>ara-600 delta (gpt-lac)5 relA1 spoT1 thi-l/</td>
<td>C. Cupples</td>
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</tr>
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<td></td>
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<td>As AB1157 but dam-13::Tn9</td>
<td></td>
</tr>
<tr>
<td>GM4252-</td>
<td>As CC106 to CC107 but mutS215::Tn10</td>
<td></td>
</tr>
<tr>
<td>GM4257</td>
<td>As AB1157 but mutS458::mTn10Kan</td>
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<td>GM8026</td>
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<td>GM8047</td>
<td>As JW177 but dam-13::Tn9</td>
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GM8051  \(dnaX36(Ts) \ thr-1 \ leuB6(Am) \ fhuA2 \ pro-33\)

\(lacY1 \ supE44(AS) \ gal-6 \ uidA2 \ hisG1(Fs) \ rfbD1\)

\(galP63 \ xylA7 \ mtlA2 \ delta \ argH1 \ rplL9(L?) \ thi-1\)

GM8056  As GM4799 but  \(dnaN159 \ zid-501::Tn10\)

GM8058  As GM4799 but  \(dam-13::Tn9\)

GM8060  As GM8058 but  \(dnaN159 \ zid-501::Tn10\)

GM8067  As GM8051 but  \(dam-13::Tn9\)

GM8096  As JW130 but  \(dam-13::Tn9\)

GM8128  GM8051/\(pdnaX^+\)

GM8137  AB1157/\(pdnaX^+\)

GM8301  As CC101 but  \(dnaN159 \ zid-501::Tn10\)

GM 8303  As CC102 but  \(dnaN159 \ zid-501::Tn10\)

GM 8304  As CC103 but  \(dnaN159 \ zid-501::Tn10\)

GM 8306  As CC104 but  \(dnaN159 \ zid-501::Tn10\)

GM 8308  As CC105 but  \(dnaN159 \ zid-501::Tn10\)

GM 8310  As CC106 but  \(dnaN159 \ zid-501::Tn10\)

JW130  As CR34 but  \(dnaE486\)

JW177  As CR34 but  \(dnaG3\)

Unless otherwise stated, strains are laboratory stocks. Abbreviations: Am, \textit{amber} mutation; AS, \textit{amber} suppressor; \(\Delta\) (delta), deletion; Fs, frameshift; mTn10, miniTn10;Oc, ochre mutation; Str, streptomycin; Kan, kanamycin; Tn9 and Tn10 encode chloramphenicol and tetracycline resistance respectively; Ts, temperature-sensitive.
medium with rifampicin and incubated at 30°C and 34°C or 37°C depending on cell viability, until colonies appeared. Media were also supplemented with 100 μg ampicillin/ml when required.

**Lac reversion assay:** CC101-107 dnaN159 derivatives were constructed by P1vir transduction using the closely-linked zid-501::Tn10 marker. CC101-106 mutS458 derivatives have been described (140) and CC107 mutS458 was constructed by P1 vir transduction. Ten cultures of these strains and the CC101-107 parental strains were grown to saturation in minimal media. For Figure 5-1, 10 ul of the culture was spotted on a minimal media plate containing lactose and Xgal and incubated at 30°C until colonies formed and papillae appeared. For Table 3, portions of the cultures were plated and incubated at 30°C on the same type of plates and blue colonies counted to calculate the reversion rate. Viable counts were determined on glucose minimal plates.

**Cisplatin and MNNG survival:** Cisplatin (Sigma) was dissolved in water and incubated at 37°C for at least 2 hours. The molar concentration was measured by taking an absorbance spectrum and reading a maximal absorbance at 301nm and dividing by the extinction coefficient, 131. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG (Sigma)) was prepared by dissolving 1mg of MNNG in 100 ul of DMSO and adding 900 ul of sterile water. Cells were grown in 10 ml L medium to an OD600 of 0.35-0.45, harvested and resuspended in the same volume of minimal salts. Varying concentrations of cisplatin were added and incubated at 30°C for 1 hour. Serial dilutions of cisplatin-exposed cells
were plated on L media plus ampicillin and incubated overnight. For MNNG, the logarithmic phase cells in L broth were exposed to various concentrations of MNNG for 10 minutes at 30°C followed by dilution for plating as described above.
Results

Spontaneous mutant frequency to rifampicin-resistance in wild type and \textit{dna} strains.

The DNA polymerase III holoenzyme complex, which includes the DnaE catalytic subunit and the beta-clamp, a product of the \textit{dnaN} gene, replicates the \textit{E. coli} chromosome together with accessory proteins, such as the DnaB helicase and the DnaG primase (122). Included in the holoenzyme are proteins responsible for beta-clamp loading/unloading such as one of the products of the \textit{dnaX} gene, the gamma subunit. The other product of the \textit{dnaX} gene, tau, is a dimer and each monomer contacts DnaE subunits on the leading and lagging strands. Tau is, therefore, responsible for maintaining the polymerase dimer configuration of the holoenzyme but in addition is also a processivity switch (122).

If the interaction between the MutS protein and the beta-clamp, a product of the \textit{dnaN} gene, is required for efficient MMR then if it is diminished, a mutator phenotype should result. We therefore tested the parental and temperature sensitive mutant strains \textit{dnaB}, \textit{dnaE}, \textit{dnaG}, \textit{dnaN}, and \textit{dnaX} for spontaneous mutation frequency to rifampicin-resistance at the permissive temperature, 30°C. The results in Table 5-2 show that \textit{dnaN} has the highest mutation frequency, a 35-fold increase, in comparison to the parental strain. Even at 34°C, where viability is reduced to about 50% of that at 30°C, a 13-fold
Table 5-2. **Mutation Frequency to Rifampcin Resistance.**

<table>
<thead>
<tr>
<th>Allele</th>
<th>Temp</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>dnaB43</td>
<td>30°C</td>
<td>1.6</td>
</tr>
<tr>
<td>dnaE486</td>
<td>30°C</td>
<td>9.6</td>
</tr>
<tr>
<td>dnaG3</td>
<td>30°C</td>
<td>0.8</td>
</tr>
<tr>
<td>dnaN159</td>
<td>30°C</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>34°C</td>
<td>13</td>
</tr>
<tr>
<td>dnaX36</td>
<td>30°C</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>37°C</td>
<td>10.1</td>
</tr>
</tbody>
</table>

The mutant frequency to rifampicin resistance was measured by simultaneously cultivating ten independent cultures of each *dna* mutant with its isogenic wildtype and expressing the result as a ratio. The average mutant frequency for the wildtype was $2 \times 10^{-8}$. Viability was the same at both temperatures for *dnaX36* bacteria but only 50% at 34°C compared to 30°C for the *dnaN159* strain.
increase in mutation frequency still occurs. This frequency is lower than expected suggesting that other lethal effects may be occurring at this temperature.

The dnaX mutant has a wildtype level of spontaneous mutagenesis at 30°C but this is increased to 10-fold at 37°C, a temperature at which full viability is retained. A strain with a holD mutation, which encodes the psi subunit of the holoenzyme, has been reported to show a seven-fold increase in spontaneous mutation frequency using rifampicin-resistance as an assay (152). Bacteria with the dnaE486 allele show a 10-fold increase in mutation frequency to rifampicin resistance (Table 5-2) confirming a previous 40-fold increase with this marker (153) and a 6-fold increase in his-4 reversion (154). The dnaB and dnaG mutants do not have a mutator phenotype (Table 5-2), as there is less than a 2-fold difference when comparing these strains with the wild type strain. We consider a two-fold difference negligible. We conclude from these results that, in general, mutations in genes specifying proteins within the holoenzyme confer a mild to moderate mutator phenotype while mutation in genes for non-holoenzyme replication proteins, show no mutator phenotype.

**Mutation spectrum of the dnaN159 strain.**

To determine the mutation spectrum in the dnaN159 strain, we have used the lacZ reversion assay described by Cupples and Miller (155, 156). The dnaN159 mutation was introduced into each of the seven tester strains and the number of Lac⁺ revertants was measured at 30°C and compared to the wildtype strains. The results are shown in Table 5-
Figure 5-1. **Reversion of lacZ alleles**

CC101-CC107 (top row), and their *dnaN159* (middle row) and *mutS215* (bottom row) derivatives.

![Image of gel electrophoresis with bands labeled wildtype, dnaN159, and mutS458.]  

**Table 5-3. Reversion frequencies (x 10^-3) of lacZ alleles.**

<table>
<thead>
<tr>
<th>Base change</th>
<th>Wildtype</th>
<th>dnaN159</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT to CG</td>
<td>172</td>
<td>188</td>
<td>1.1</td>
</tr>
<tr>
<td>GC to AT</td>
<td>286</td>
<td>220</td>
<td>0.8</td>
</tr>
<tr>
<td>GC to CG</td>
<td>6</td>
<td>8</td>
<td>1.3</td>
</tr>
<tr>
<td>GC to TA</td>
<td>4620</td>
<td>316</td>
<td>0.07</td>
</tr>
<tr>
<td>AT to TA</td>
<td>206</td>
<td>1430</td>
<td>7</td>
</tr>
<tr>
<td>AT to GC</td>
<td>12</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>6G to 7G</td>
<td>13300</td>
<td>402</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Measurements of lacZ reversion were carried out at 30°C.
There is a 7-fold increase in AT to TA transversions which may be due to increased SOS induction in this strain (153, 157). There are also reductions in GC to TA transversions (14-fold) and +1 frameshifts (33-fold) in the dnaN159 strain relative to wildtype. These changes are not those expected in MMR-deficient strains which show increases in transition and +1 and -1 frameshift mutations (140, 155, 156). We conclude that the increased spontaneous mutation frequency in the dnaN159 mutant is not due to diminished MMR.

Survival of dna mutants after treatment with cisplatin.

_E. coli dam_ mutants are more sensitive to cisplatin and MNNG than wildtype (21, 69). We constructed _dam_ derivatives of the _dna_ mutants and tested each for sensitivity to these cytotoxic agents. Inactivation of MMR by mutation in _mutS_ or _mutL_ in a _dam_ background renders the double mutants as resistant to cisplatin and MNNG as wildtype. By combining the _dna_ mutations with _dam_, the effect of MMR can be monitored.

The survival of the _dna_ mutants after exposure to cisplatin was variable and can be classified into two groups. The first group comprises _dnaB43_, _dnaG3_ and _dnaN159_ which are slightly more sensitive to cisplatin than wildtype at 30°C (Figures 5-3A, 5-4A, 5-5A, 5-6A). These mutant strains do not show additional sensitivity to cisplatin when combined with _dam-13_ mutant.

In the second group are the _dnaE486_ and _dnaX36_ mutants. Both are as sensitive to cisplatin as a _dam-13_ mutant and the double mutant _dnaE486 dam-13_ is more sensitive
than either parent (Figure 5-2A). Survival was determined at 30°C for the dnaE486 strain and 37°C for the dnaX36 mutant. The sensitivity of the dnaE486 strain to cisplatin is in marked contrast to that for MNNG to which the strain has the same survival as wildtype (Figure 5-2B). Although the dnaX36 dam-13 double mutant is viable at 30°C, it is not viable at 37°C in contrast to both parental strains. We assume the inviability is due to inability to repair all the DSBs formed at this temperature.

An unusual feature of DnaX is that when expressed from a multicopy plasmid at 30°C (data not shown) or 37°C, the dnaX36 host strain bearing it becomes sensitive to cisplatin (Figure 5-6A), to the same degree as a dam mutant, but remains resistant to MNNG (Figure 5-6B). No sensitivity to cisplatin was detected when the same plasmid is expressed in a wildtype strain (Figure 5-6A).

### Survival of dna mutants after treatment with MNNG

In contrast to cisplatin, the response of the dna mutants to MNNG is more complex. The dnaE486 mutant shows the same survival after MNNG treatment as wildtype (Figures 5-2B, 5-6B) while the dnaB43, dnaG3, dnaN159 and dnaX36 mutants are more sensitive (Figures 5-3B, 5-4B, 5-5B). When combined with dam-13, there is no additional increase in sensitivity for dnaB43 (Figures 5-5B, 5-6B) but an increase in sensitivity is exhibited with dnaG3 and dnaE486 (Figures 5-2B, 5-4B).
Figure 5-2: Survival of wildtype, dnaE486, dam-13 and dam-13 dnaE486 strains after treatment with cisplatin and MNNG

Fig. 2

A. Survival (% Control) vs. Cisplatin (μg/ml) 
B. Survival (% Control) vs. MNNG (μg/ml)

- Wildtype
- dnaE486
- dam-13
- dam-13 dnaE486
Figure 5-3. Survival of wildtype, dnaN159, dam-13, dam-13 dnaN159 and dam-13 dnaN159 mutS458 strains after treatment with cisplatin and MNNG.

Fig. 3

A. 

B. 

Fig. 4

A. 

B. 

Figure 5-4. Survival of wildtype, dnaG3, dam-13 and dam-13 dnaG3 strains after treatment with cisplatin and MNNG.
Figure 5-5. Survival of wildtype, *dnaB43*, *dam-13* and *dam-13 dnaB43* strains after treatment with cisplatin and MNNG

Fig. 5

**A.**

- **B.**

Fig. 6

- **A.**

- **B.**

Figure 5-6. Survival of *dnaX36* at 30°C and 37°C, *dam-13* and *pdnaX* strains after treatment with cisplatin and MNNG
With *dnaN159*, the *dam-13 dnaN159* double mutant is more resistant to MNNG than the *dam-13* strain alone (Figure 5-3B). This result is very similar to that obtained when MMR is compromised by null mutations in the *mutS* or *mutL* genes in a *dam* mutant leading to MNNG resistance to the same level as a wildtype strain. Loss of MMR prevents recognition and processing of O\(^6\)-meG base pairs and likely prevents the subsequent lethal futile cycling from ensuing (21). To monitor the effect of MMR on *dnaN159 dam-13* survival, a *dnaN159 dam-13 mutS458* triple mutant was constructed. The survival of the double and triple mutants after MNNG exposure was the same (Figure 5-3B). This result indicates that the increased resistance of the *dnaN159 dam-13* strain to MNNG is not due to impairment of MMR.
Discussion

One goal of this work was to examine if the interaction between the DnaN159 clamp and MutS was diminished or not. The results in Figure 5-1 and Table 5-3, indicates that the interaction, if indeed is necessary, is not affected in the mutant strain, as the mutation spectrum is not the same as for a mutS mutant (Figure 5-1). The dnaN159 mutant displays constitutive SOS induction and this is the likely explanation for the increase in AT to TA transversions (157). The surprising reduction in +1 frameshift mutations and GC to TA transversions could be due to slower DNA replication in the dnaN159 mutant resulting in more efficient repair of replication errors (frameshifts) and spontaneous oxidative lesions (8-oxoguanine) (158). For the latter, it is also possible that SOS-induced translesion polymerases in the dnaN159 strain efficiently bypass this modified base while in the wildtype it is mutagenic when replicated by DNA polymerase III holoenzyme.

If the MutS-beta clamp interaction is important for MMR sensitization, then an increase in resistance to cisplatin and MNNG might be expected in the dam-13 dnaN159 mutant. At the permissive temperature, there was no difference in survival between the double mutant and dam-13 strain to cisplatin (Figure 5-3). The dam-13 dnaN159 strain is more resistant to the cytotoxic effect of MNNG than dam-13 bacteria which could indicate a reduction in MutS-beta clamp interaction. However, this increased resistance is
not MutS-dependent as the triple dam-13 dnaN159 mutS458 mutant is as sensitive as the double and does not restore wildtype levels of resistance (Figure 5-3). The results above indicate that MutS-beta clamp interaction is not altered in the dnaN159 strain and thus the fidelity of MMR is intact.

MMR in mammalian cells or E. coli dam mutants increases the cytotoxic effects of cisplatin and MNNG. Although the phenotypic effect of both agents on dam (drug sensitivity) and dam mut (drug resistance) strains are the same, the underlying molecular mechanism for each agent could be different. This was another goal of this work; the contrasting results for cell survival of dna mutants exposed to these agents argue that MMR processing of cisplatin adducts and O⁶-meG base pairs is, indeed, different. Our recent observation that the plasmid-borne mutS800 mutation, which prevents MutS tetramer formation, sensitizes cells to MNNG but not to cisplatin is consistent with this conclusion (142).

The dnaE486 strain offers an example of the difference between cisplatin and MNNG exposure. The explanation for the differential response of dnaE486 these agents is undoubtedly that a cisplatin intrastrand crosslink is a block to replication while an O⁶-meG base pair is not. The sensitivity of the other dna mutants (which are all involved in lagging strand synthesis) to cisplatin is probably also related to replication blockage but the higher sensitivity of the dnaE486 mutant suggests that blocking leading strand replication is potentially more dangerous to the cell than blocking lagging strand
replication. Replication forks blocked by cisplatin lesions can either disintegrate or be processed, in each case forming double-strand breaks in DNA which require recombination and primosomal proteins for their repair (85, 86, 101).

Mutant (dnaX36) bacteria containing the DnaX overproducing plasmid are fully viable at 43°C but show increased sensitivity to cisplatin, but not to MNNG, at both 30°C and 37°C. This result suggests that the holoenzyme complex may be slightly defective under these conditions or that the dnaX gene products regulate the composition of the holoenzyme. That overproduction of DnaX in the wildtype does not lead to cisplatin sensitivity, suggests that either the former possibility is correct or that the dnaX36 mutation is dominant for cisplatin sensitivity but not temperature-sensitivity.

In a dam cell, MMR is not confined to the region behind the replication fork as in wildtype. Because the MutH protein can use unmethylated DNA as a substrate, mismatches occurring in unreplicated DNA are also subject to repair. MMR at these sites could generate DSBs by MutH action at the same GATC sequence on opposite strands, or as a consequence of replication fork collapse at the gap or nick during the exonucleolytic phase. The constant generation of DSBs results in recombination capacity in the cell being at, or near, its maximum (88). The additional breaks caused by cisplatin damage leads to saturation of recombination capacity resulting in unrepaired DSBs and cell death. This interpretation is supported by increased cell survival of dam mutS mutants because MMR is inactivated. This is directly related to the reserve recombinational capacity now
available to repair any damage, such as the DSB, induced by cisplatin treatment. We have recently demonstrated dose-dependent, cisplatin-induced DSBs in dam mutants in agreement with the above scenario (159).

The increase in sensitivity to both agents of the dnaE486 dam-13 mutant (Figure 5-2), in contrast to either parent alone, is likely the result of the cell attempting to repair the large number of endogenous DSBs created by MMR as well as those produced by cisplatin and, presumably by MNNG. The dam-13 dnaG3 strain showed greater sensitivity to MNNG, but not cisplatin, than either parent (Figure 5-4). This result again demonstrates a difference between cisplatin and MNNG. Since the DnaG primase is involved in lagging strand synthesis, it may be that it promotes the preferential insertion of thymines opposite O6-meG residues to promote futile cycling (see below) by MMR on the lagging strand thereby interfering with fork progression. For cisplatin adducts, which do not promote futile cycling by DNA polymerase III, the gap produced in the lagging strand can be repaired by recombination after passage of the replication fork.

Although MMR can recognize lesions created by both cisplatin and MNNG, there is a distinct divergence in the mechanism necessary to repair the damage, as exemplified by the differential sensitivities of the dna mutants to these agents. The effect on DNA replication could be at the level of the replication fork during chromosome replication either by replicative or by-pass polymerases and/or during the re-synthesis step in MMR, which is catalyzed exclusively by DNA polymerase III holoenzyme (126). MNNG
damage recognized by MutS will likely primarily follow the futile cycling model where $O^6$-meG paired with cytosine or thymine is recognized as a mismatch and excised. Since no "good" match exits, a futile cycle of removal and synthesis ensues behind the replication fork (21). Fork progression is blocked, however, and it becomes susceptible to reversal and/or disintegration requiring recombinational repair to restore an active fork. It is unlikely that futile cycling occurs with cisplatin lesions, as progression of the replicative polymerase and DNA polymerase I would be blocked by intrastrand crosslinks. We have shown previously that recombination is essential for repair of cisplatin lesions (10) and that MMR, \textit{in vitro}, inhibits the initiation of recombinational repair with platinated substrates by interfering with the action of RecA (33). We have also shown that cisplatin is highly recombinogenic, but that MNNG is less so (10, 80). At present it is unclear how recombination substrates are generated at a greater frequency by cisplatin compared to MNNG, as lesions produced by both should lead to replication fork stalling and reversal, although direct fork blockage is probably more efficient with cisplatin adducts than $O^6$-meG. That \textit{dnaN159 dam-13} bacteria are no more sensitive to cisplatin than the \textit{dam-13} strain argues that the step at which such recombination substrates are generated must be prior to the polymerase III holoenzyme re-synthesis of gaps produced by exonucleolytic action after MutH incision at a GATC site. If so, this implies that in \textit{dam} mutants, cisplatin intrastrand crosslinks in unreplicated DNA are also a source of recombination substrates as well as those at stalled replication forks. If these gaps are long lived, a replication fork passing through them will collapse provoking recombinational repair. In contrast, MNNG lesions at the replication fork are the primary
site of action of the MMR system to promote sensitization through futile cycling. In unreplicated DNA, O\(^6\)-meG-cytosine pairs can also be sites for futile cycling which may lead to collapse of replication forks passing through them but these sites may be less frequent or have a shorter half-life than those for cisplatin. We are currently testing these ideas.

Acknowledgements

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CHAPTER VI

FINAL COMMENTS AND FUTURE DIRECTIONS

Recombination is an important process to repair DNA damage in the cell. Previous to the work presented here, it was known that recombination was as essential as NER to tolerate cisplatin damage and that the types of damage induced by cisplatin invoking recombination was likely DSG/DSB formation. In Chapter 2, we identified the gene products required for spontaneous and cisplatin induced recombination using the duplicated inactive lac operon plate assay. Recombination is probably induced following stalling or collapse of replication forks, through naturally occurring circumstances or through cisplatin blockage, ultimately leading to DSBs. Spontaneous recombination requires the RecBCD pathway for repair and DSBs formed by chromosome replication are the likely cause for such spontaneous recombinants. This is not surprising as about twenty percent of replication forks never complete chromosome replication without interruption (87). Stalling or collapsing of forks from various types of normally occurring DNA damage can initiate recombination through this pathway also.

In contrast, cisplatin-induced recombination requires the RecFOR pathway, in addition to the RecBCD pathway identified in the spontaneous induced recombinants. As cisplatin lesions are blocks to the replication machinery, fork stalling is imminent and the mechanism to invoke recombination is dependent on whether the lesion is on the leading
or lagging strand. Lesions on the leading strand can cause fork stalling, regression, and ultimately lead to DSBs if encountered by a subsequent fork for example, while lesions on the lagging strand can initiate DSG repair after fork stalling. The requirement for RecFOR and RecBCD pathways exemplifies the efficiency of the repair system, as the RecBCD pathway is mainly responsible for repair of leading strand lesions and RecFOR for gap repair in the lagging strand (Chapter 2). Cisplatin-induced and spontaneous recombination requires both RecBCD and PriA, to process regressed forks and to reload the replication machinery to continue synthesis respectively, thus providing the cell with a tolerance mechanism for cisplatin lesions.

Understanding the requirements for cisplatin-induced recombination enabled examination of a broader question, namely, what is the mechanism of cisplatin toxicity? (Chapter 3). As human cells and dam mutants are sensitive to treatment with cisplatin, mutations in MMR render them as resistant as a wild type cell. Thus it is implied that MMR proficiency must aid in the toxicity of cisplatin, but the mechanism was not well understood. We proposed that the mechanism of MMR sensitization to cisplatin was analogous to the mechanism for MMR prevention of interspecies recombination. As MutS binds mismatches produced by the attempt to recombine DNAs from two related species to abort recombination, we found that MutS also bound cisplatin lesions to prevent recombination in the same manner. Relatively few cisplatin lesions in the DNA reduced RecA-mediated strand exchange and the addition of MutS blocked recombinational repair. When MutS was added to an already progressing reaction,
further product formation in the RecA strand exchange reaction was inhibited, and a larger amount of intermediate structures was present. From this we concluded that MutS can inhibit recombination at the step after RecA initiates strand exchange, binding to the platinated lesion in the heteroduplex region formed at this point, thus preventing recombination and causing cell death. In the absence of MutS, strand exchange can occur opposite the lesion albeit at a slower rate, leaving it for repair by NER.

The mechanism by which cisplatin-induced DSBs are formed in dam mutants is still unknown. Recent work using pulse-field gel electrophoresis has confirmed that in dam mutants it is DSBs that are induced by cisplatin (159). As mutations disabling MMR renders dam cells resistant to cisplatin, MMR invokes cisplatin cytotoxicity and must play a role in enabling DSB formation in dam mutants exposed to cisplatin. Figure 6-1 is our proposed mechanism for MMR provoked DSB formation and repair in the presence of cisplatin lesion (159). As MutS can initially recognize the platinated crosslink, it will recruit MutL and MutH to the lesion. MutH incision and subsequent exonucleolytic digestion creates a gap, which may or may not remove the adduct, as it depends on the strand where the adduct resides. In dam mutants such gaps can occur on either strand. These gaps can lead to replication fork collapse if encountered by another DNA polIII holoenzyme. Regardless of which strand contains the adduct, replication fork collapse upon encountering the gap produces a molecule with a double stranded end, a substrate for RecBCD (center of Figure 6-1). After encountering Chi, a 3’ single stranded tail is formed, which allows RecA to load and promote strand exchange,
In \textit{dam} mutants mismatch removal can occur on either DNA strand. On the left, the strand with the cisplatin adduct is excised and on the right, the strand without the lesion. In both cases mismatch-directed excision or re-synthesis is incomplete when a new replication fork encounters the gap and collapses. This creates a double-stranded end, which can be acted upon by RecBCD (center panel), which after encountering Chi, promotes RecA strand transfer, followed by RuvAB helicase action and RuvC cleavage, thus restoring the replication fork. PriA can reload the replisome to restart replication. The bottom strand of the gapped structure on the right, remaining after fork collapse cannot be extended by polymerase as in the left panel, due to the presence of the lesion on the complementary strand. RecFOR proteins can catalyze RecA loading at the gap and after RuvABC action, fork restoration is complete and PriA reloading can occur. RecA strand transfer can be inhibited by MutS where indicated.

\begin{itemize}
\item \textbf{RecBCD}
\item \textbf{MutS -I +}
\item \textbf{RecA}
\item \textbf{RuvABC}
\item \textbf{PriA}
\end{itemize}
allowing for restoration of the replication fork and reloading of the replisome by PriA. If the adduct is on the strand subject to exonucleolytic digestion, polymerase extension and ligation forms an unbroken DNA strand which may or may not contain the lesion depending on the extent of excision (left of Figure 6-1). If the adduct is still present, it can be bound by MutS for another attempt by the MMR system or removed by NER. Template strand lesions cannot be converted to duplex DNA after fork collapse because the adduct prevents polymerization on the complementary strand (right of Figure 6-1). However, this damage can be repaired by gap repair, requiring the RecFOR pathway. Once acted upon by RecFOR, RecA can load and initiate recombination to restore the fork as described above. Regardless of the strand location of the adduct, the model insists upon invoking recombinational repair of a DSB. As recombinational repair of collapsed forks from cisplatin lesions requires the strand exchange activity of RecA and that MutS and MutL (in vivo) inhibits that activity when a substrate contains a platinated substrate, this can explain MMR-dependent sensitization of dam mutants to cisplatin, as indicated by the binding of MutS in the figure. This model using the dam mutants is likely the most relevant model, as the circumstances surrounding the damage induced by DNA are occurring in unreplicated DNA. As most of the chromosome in the cell is not being replicated, a large number of breaks are being induced through this mechanism. In replicating cells the RecBCD and FOR pathways are still equally important as any DSBs or DSGs formed from fork damage require repair from those pathways also. The experiments in Chapter 2 verify the need for these pathways in replicating cells and in wildtype cells, as the experiments in that chapter and in the original studies examining
the requirement of recombination were executed in a wildtype genetic background (10). In addition, MMR confined to just behind the fork in a "futile cycling" manner would also follow DSB or DSG repair and would occur more infrequently as it has a dependence on replication.

A puzzling conclusion from the platinated strand exchange experiments was that MutL had no effect in the \textit{in vitro} system, but is implicated in conferring cisplatin resistance to \textit{dam} cells \textit{in vivo}. We presumed that the number of lesions was a factor and that \textit{in vitro} the number of cisplatin lesions is sufficiently handled by MutS without needing the potentiating effect of MutL, which is required \textit{in vivo}. This also makes an assumption that a cisplatin lesion is likely visualized differently than a mismatch, as the strand exchange reaction can tolerate many more mismatches than platinum adducts to form the same amount of product. As an alternate explanation for the dispensability of MutL, we proposed there was a factor missing \textit{in vitro} that is available \textit{in vivo}, such as the beta clamp of the polymerase III holoenzyme. As the clamp has been shown to interact with MutS and the holoenzyme is required for re-synthesis during repair \textit{in vitro}, this prompted us to design the experiments examining the sensitivity of \textit{dna} mutants to cisplatin and MNNG, beginning with the experiments with \textit{dnaN} (Chapter 5). If the clamp was important for sensitization or repair by MMR, then an increase in resistance to cisplatin and MNNG would be expected. Such a result was not obtained. We also examined other \textit{dna} strains with mutations in both holoenzyme and non-holoenzyme associated genes for sensitivity to cisplatin and MNNG, as their mode of sensitization
may be different. These replication mutants did not display the same sensitivity as each other upon exposure to both agents. Most striking was the result for the *dnaE* mutant, which showed sensitivity to cisplatin but not MNNG. That both *dnaG* and *dnaB* mutants, involved in lagging strand synthesis were also sensitive to cisplatin, but the *dnaE* mutant is much more sensitive suggesting blockage in the leading strand is more detrimental than in the lagging strand. The general lack of sensitivity of these mutants to MNNG implies that such damage is likely processed fully by MMR and recombination is invoked only when forks stall at a futile cycling of O\(^6\)-methylguanine in progress. As cisplatin lesions are polymerase blocking lesions, the effect with *dnaE* is not surprising. The distinct difference of the other *dna* mutants when compared to the *dnaE* mutant in their sensitivity to cisplatin, implies that it is the polymerase itself that is blocked and the holoenzyme accessory proteins have a more minimal effect. That the same mutants have a more mild sensitivity to MNNG, confirms that a mismatch and a cisplatin lesion are processed differently and pose a unique set of problems for the cell in regards to replication and repair.

As the interaction of the beta clamp with MutS is a relatively new finding, there are many unanswered questions about the importance of this interaction. We have obtained a set of *mutS* mutant strains with point mutations reducing the interaction with the clamp from our collaborators, the lab responsible for this discovery (135). As the studies with these mutants thus far are purely interaction assays, we intend to study the effect of these mutations *in vivo* by determining sensitivity to cisplatin and MNNG in a
"dam" background. If these mutants are resistant to MNNG, this would be the first in vivo evidence that confirms the necessity of a MutS/clamp interaction for mismatch repair. These studies act as a complement to the *dnaN* experiments, in that enables examination of the importance of the clamp in a different capacity.

The hypothesis that cisplatin and MNNG lesions are processed differently was further explored by the experiments in Chapter 4, with the *mutSΔ800* mutant. The plasmid expressed *mutSΔ800* mutant displayed a differential sensitivity to cisplatin and MNNG in that it was as resistant to cisplatin as a null mutant and as sensitive to MNNG as a wildtype *mutS* strain. As Δ800 is restricted to only dimer formation, this implied that recognition of cisplatin lesions requires greater than dimers of MutS, hence the cisplatin resistant phenotype. This was confirmed with our band shift studies where MutSΔ800 did not recognize the 1,2- intrastrand simple crosslink and has reduced affinity for the compound lesion when compared to wild type MutS. Interestingly, MutSΔ800 can bind mismatches and prevent strand exchange between the M13 and fd phage genomes at a level close to that of wild type MutS. These MutS studies confirmed that for cisplatin lesions, the mechanism for mutation avoidance by MMR is not analogous to that for antirecombination. Antirecombination with cisplatin likely requires tetramers of MutS while repair of mismatches is efficient with dimers of the protein. Thus binding of a mismatch by MutS is not analogous to binding of a cisplatin adduct. As these studies were performed with plasmids, we have constructed a *mutSΔ800* mutation on the
chromosome and are currently repeating these experiments to confirm these results in single copy.

The studies presented in this work provide a framework for many future experiments. MMR can recognize lesions created by cisplatin and MNNG, but there is an obvious divergence in the mechanism necessary to repair the damage, as exemplified by the differential sensitivities of the mutants used in this work. MNNG damage recognized by MutS behind the replication fork will likely follow the futile cycling model and MMR will continue to excise and re-synthesize the inappropriate base match until recombination is induced to repair the stalled fork. MutS also recognizes cisplatin lesions and blocks strand exchange in vitro, and recombination is essential for repair of cisplatin damage. Where MMR repair/recognition of cisplatin lesions ends, and recombinational repair of such lesions begins is not known. We presume at some point after MutS recognition of the lesion, a substrate is created that is highly specific for recombinational repair proteins. As the dam dnaN mutant was no more sensitive to cisplatin than dam alone, this argues that the step at which recombination substrates are generated are prior to holoenzyme re-synthesis, for which the clamp is required. A possible point for this to occur is during or post- exonuclease digestion of the DNA after MutH incision in MMR. Using the oligonucleotide containing the centrally located G-G crosslink from our band shift assays, we will use commercially available exonucleases to determine whether digestion can proceed through the crosslink, visualized by electrophoresis of reactions with and without exonuclease. If the exonucleases arrest upon encountering the
crosslink, this could generate a substrate for recombination. If the exonucleases proceed through the crosslink, this suggests that the MMR exonucleases are either not involved, or they are as capable of degrading cisplatin lesions as they are mismatched bases. In addition we have obtained mutants of all the MMR exonucleases and will test each singly and in combination with the other MMR exonucleases to determine sensitivity to cisplatin and MNNG. These results could give information as to where MMR and recombination diverges as well as providing additional information about how a DSB is generated by cisplatin. There is still much to be studied in regards to the mechanism of cisplatin toxicity and the roles of MMR in drug resistance and we hope the studies in this thesis, in addition to the new experiments proposed, will inspire future work in this area.
APPENDIX I

PROTEIN PURIFICATION- A DETAILED METHOD

For the strand exchange method in Chapters 3 and 4, the purification of RecA and MutSΔ800 protein was briefly described and will now be elaborated upon, step by step in this appendix.

RecA protein was purified from strain GM7487 (precA430/F-lacI' lacZΔM15 pro
$A^+B^+/P90C\ (ara\ \Delta(lac-pro)13)$ (as described with modifications). This strain contains the recA gene under an IPTG inducible promoter for expression. On the first day the cells were grown by inoculating a fresh colony from a bacterial plate into 100 mls of Brain Heart Infusion (BH) medium and grown overnight at 37°C. The next morning, 10 ml of overnight culture was used to inoculate six individual one liter cultures of twice concentrated BH medium containing ampicillin (100 μg/ml). The strain was allowed to grow at 37°C for approximately 2-3 hrs, until the cells reached an OD$_{600}$ of 0.5-0.75. Subsequently, the cells were induced with 1mM IPTG and grown an additional 2 hrs before harvesting. Induction was checked by SDS-PAGE analysis comparing the induced to the uninduced culture (data not shown, as there is no discernable difference between the two cultures). The cells were harvested in 1 L bottles in a Sorvall RC-3 centrifuge for 30 minutes at 4K. The cell pellet was resuspended as a cell paste in 25 ml of a cold 25% sucrose/0.25 M Tris pH 7.5 solution, and the centrifuge bottles washed
with an additional 25 ml total of fresh sucrose/Tris solution. The paste was transferred to 50 ml conical tubes and frozen in liquid nitrogen. At this time, it is important to prepare all the solutions required for the proceeding cell lysis steps as well as all the buffers needed for FPLC. Table A1-1 lists each buffer and its components for the RecA purification steps.

To initiate the cell lysis process, the cell paste was thawed at 4°C on ice. Once the paste was completely thawed, it was diluted with an equal amount of sterile water. Lysozyme (5.4 ml of a 10 mg/ml solution) was added to the paste and stirred on ice for 1 hr. Subsequently 9.3 ul of 14.4 M BME and 1.0 ml of 0.5 M EDTA was added to prevent any oxidation reduction reactions involving the proteins in the paste which may take place during lysis. After stirring for 30 min., another 5.4 ml of lysozyme was added and stirred for an additional hour. Next, 46ml of a 3% Brij-58/50 mM Tris pH 7.5 solution was added to the cell lysate and continuously stirred for 30 min. to enable the detergent to continue the lysis process. The lysate was then spun at 13K for 90 minutes in an RC-5 centrifuge in 2-250 ml bottles. The supernatants were combined and 32 ml of a 10% PEI (polyethyleneimine) solution was slowly added 5 ml at a time, stirring rapidly by hand, to facilitate the binding of nucleic acids to the PEI and to prevent the PEI from binding to itself. This step is critical, as RecA is a DNA binding protein and will be associated with the nucleic acids at this step. The PEI/lysate solution was stirred for an additional 30 minutes and then centrifuged for 20 min. at 13K. Since the nucleic acids and RecA are bound by the PEI, the pellet was saved, and to it 100 ml of R buffer +150mM ammonium
sulfate was added. The pellet was homogenized with a hand-held blender and stirred on ice for 30 min. The solution was centrifuged again for 15 min. at 13K and the pellet recovered. An additional 75 ml of R buffer +300 mM ammonium chloride was added to the pellet and homogenized again to resuspend it efficiently. After stirring on ice for 30 min., and centrifuging again as in the last step, the supernatant was saved. The addition of ammonium sulfate in incremental concentrations allows for dissociation of the proteins from the nucleic acids bound in the PEI pellet. RecA will only elute from the pellet at the higher ammonium sulfate concentration. For each ml of supernatant recovered after the final centrifugation step, 0.32 g of solid ammonium sulfate was added to enable precipitation of the proteins present in solution. The solution was stirred overnight at 4°C.

The ammonium sulfate precipitated solution was centrifuged for 45 min. at 12K. The resulting pellet was dissolved in 20 ml of R buffer and conductivity monitored using a Fisher conductivity meter. The protein was continually diluted until the conductivity reading reached between 8 and 10 μS.

The next step in the purification process was to load the lysate onto a 60 ml Q sepharose column. Q sepharose resin is used as an anion exchange resin as well as for size exclusion and replaces the DE-52 and Sephacryl S-1000 columns originally used to purify this protein. The Q sepharose column must first be equilibrated by running four column volumes of R buffer + 20mM ammonium chloride through the column, after
which the sample can be loaded onto the column either using the loop or manually using a peristaltic pump. After loading, the column was washed with 2 column volumes of the same buffer to eliminate any proteins that do not bind the column. The wash fractions were collected in 50 ml conical tubes. An increasing linear gradient from 200 mM to 500 mM ammonium chloride was used to elute the protein from the column in 12 ml fractions. The FPLC trace is shown in Figure A1-1. The collected fractions were analyzed by SDS page and fractions containing RecA were pooled (Figure A1-2). The pooled fractions were precipitated overnight with ammonium sulfate as done previously.

The following day the precipitated protein was spun down as previously described and loaded onto a PD-10 desalting column. After elution, this step was followed by loading the fractions containing RecA onto a pre-swelled single-stranded DNA cellulose column (Sigma), equilibrated with 25 mM NaCl in R buffer and eluting with a solution of R buffer +500 mM NaCl and 2 uM ATP (Figure A1-3). The fractions containing RecA were pooled and concentrated using Millipore protein concentrators with a molecular weight cutoff of 10K. Analysis of the purified protein by SDS-PAGE showed no visible contaminants (Figure A1-4). The concentration of RecA was determined by the ninhydrin protein assay (128) and activity determined by strand exchange, as in order to be active, RecA must bind to DNA and hydrolyze ATP, both also required for function in strand exchange.
Table A1-1. **Buffers needed for RecA purification.**

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R Buffer (1L)</strong></td>
<td>need about 8 L for purification filtered</td>
</tr>
<tr>
<td>20 mM Tris HCl pH 7.5 (2.42 g Tris/L)</td>
<td></td>
</tr>
<tr>
<td>0.1 mM EDTA (200 ul of 0.5 M/L)</td>
<td></td>
</tr>
<tr>
<td>5% glycerol (50 ml/L)</td>
<td></td>
</tr>
<tr>
<td>5 mM BME (350 ul/L) - add fresh when needed</td>
<td></td>
</tr>
<tr>
<td><strong>R Buffer + salt</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Lysis</strong></td>
<td>150 mM Ammonium Sulfate - 1.98 g/100ml - need 200 ml</td>
</tr>
<tr>
<td>300 mM Ammonium Sulfate - 3.96 g/100ml - need 200 ml</td>
<td></td>
</tr>
<tr>
<td><strong>Q Sepharose column</strong></td>
<td>1 M Ammonium Chloride- 53.49 g/L - need 1 L filtered</td>
</tr>
<tr>
<td><strong>ssDNA cellulose column</strong></td>
<td>25 mM Sodium Chloride- 1.46 g/L - need 1 L</td>
</tr>
<tr>
<td>500 mM Sodium Chloride- 29.22 g/L - need 0.5 L</td>
<td></td>
</tr>
<tr>
<td><strong>3% Brij 58/Tris</strong></td>
<td>(100ml)</td>
</tr>
<tr>
<td>50 mM Tris (0.6 g/100ml) pH 7.5</td>
<td></td>
</tr>
<tr>
<td>Brij 58 (3 g/100 ml)</td>
<td></td>
</tr>
<tr>
<td><strong>10% PEI</strong></td>
<td>(100ml)</td>
</tr>
<tr>
<td>10 ml of PEI solution into 90 ml of water</td>
<td></td>
</tr>
</tbody>
</table>
Figure A1-1. **Q sepharose Chromatogram.**

Chromatogram showing the elution of RecA protein (ml of gradient eluent) as a function of absorbance at 280 nm.
Figure A1-2. **Elution of RecA protein from Q sepharose column.**

The cell lysate from day one was loaded onto a Q sepharose column for further purification. Gels containing representative fractions (10 ul from 12 ml) collected from the gradient elution from the Q sepharose column. M: marker, X: wash fractions B-E: 12 ml fractions from the elution. Fractions D9-E2 contain RecA protein and were pooled and precipitated.
Figure A1-3. **Elution of RecA from an ssDNA cellulose column.**

RecA precipitated from the Q-sepharose step was loaded onto a ssDNA cellulose column and precipitated using 2 mM ATP. An aliquot of 10 ul was taken during each step to monitor the elution of RecA from the column. M-marker, ppt- load, FT, flow through, W- wash fractions 1-8, B- elution fractions containing ATP, HS- high salt wash. RecA elutes mostly in the B fractions, which were pooled and concentrated.
Figure A1-4. **Purity of the RecA protein**

The gel shows varying concentrations (determined by absorbance spectroscopy) of RecA protein after all purification steps. The protein is >95% pure. Exact concentration of the protein was determined using a ninhydrin protein assay.
MutSL800 was purified from strain GM7854 (pmutSA800/BL21(lambdADE3)) using modifications of the method previously described by Su et al. (31). The strain was grown by picking a colony from a freshly transformed plate and inoculating into 100 ml of BH medium plus ampicillin (100 µg/ml) and growing overnight. The next morning, 10 ml of overnight culture was used to inoculate six individual one liter cultures of BH media. The cultures were grown at 37°C to an OD₆₀₀ of 0.8 and then shifted to 26°C for induction with 1 mM IPTG. The cells were grown an additional 3 hrs at 26°C before harvesting by centrifuging at 4K for 30 minutes. Induction was checked by SDS-PAGE analysis, but there was no noticeable difference between induced versus uninduced cultures (data not shown). The cell pellets were frozen under liquid nitrogen. All buffers used for purification are listed in Table A1-2.

To initiate lysis, each of the six pellets were thawed and resuspended in 15 ml of buffer A. The cells were passed through a microfluidizer (Microfluidics Corp.) twice and then centrifuged at 15K for 30 min. The supernatant was treated with 42 ml of 30% weight/volume streptomycin sulfate and stirred on ice for 45 min. Ammonium sulfate (36 g) was added over a 20 min. period to precipitate the proteins and left to stir on ice for an additional 20 min. This solution was centrifuged at 15K for 30 min. and the pellets resuspended in a total volume of 10 ml of buffer A. This was then diluted 1:10 with a solution of buffer A containing 25 mM KCl. The resulting solution at this step was loaded onto a 30 ml heparin agarose column equilibrated with 50 mM KCl in buffer A (Amersham Pharmacia) and eluted with a linear gradient of 100-400 mM KCl. The
resulting chromatogram is shown in Figure A1-5. In addition, a sampling of the collected fractions was checked by SDS-PAGE to monitor the elution of MutSΔ800 (Figure A1-6), as the chromatogram shows a very diffuse peak which makes it difficult to determine which fractions contain the protein. The fractions containing MutS were pooled and subsequently loaded onto a pre-equilibrated ceramic hydroxylapatite (CHT) column (Sigma). The CHT beads were swelled in a 0.2 M potassium phosphate solution and a 60 ml column was packed by gravity flow, as pressure applied by the FPLC for packing will crush the beads. The column was washed with a solution containing buffer B+ 0.2 M KCl and the sample applied using the loop. The sample was washed with 2 column volumes of the same buffer and eluted using a linear concentration gradient of 20 mM-120 mM KCl. This produced a single MutS peak eluting at 50-70 mM KCl (Figure A1-7). Samples of selected fractions were also monitored by SDS-PAGE (Figure A1-8). Wild-type MutS protein elutes as two peaks, one containing the active protein and one containing the protein with significantly less activity, therefore the elution profile of the dimeric mutant MutSΔ800 protein is quite different. Fractions containing MutSΔ800 were pooled, concentrated using a Centriprep column (Millipore) and frozen at -75°C (Figure A1-9). The protein was at least 95% pure as determined by SDS-PAGE analysis. Exact protein concentration was determined by ninhydrin analysis (128). Activity of the protein was determined by band shift assays using homo- and heteroduplex DNA substrates with and without ATP, as ATP inhibits binding of MutS to heteroduplex DNA containing a mismatch (data not shown).
Table A1-2. **Buffers for MutSA800 purification.**

All Buffers are filtered before use

**Potassium Phosphate buffer**
Solution A- 0.4 M KH$_2$PO$_4$
Solution B- 0.4 M K$_2$HPO$_4$

For a pH 7.4 solution of 0.02 M KPO$_4$, 243 ml of Solution B + 57 ml solution A + 300 ml of water are measured in graduated cylinders and mixed in solution.

**Buffer A (need 3 L)**
0.02 M KPO$_4$ pH 7.4
1 mM EDTA
1 mM PMSF
10 mM BME

**Buffer B (need 3 L)**
0.02 M KPO$_4$ pH 7.4
0.1 mM EDTA
1 mM PMSF
10 mM BME

**Buffers A and B plus salt addition for FPLC elution**

Buffer A + 0.025 M KCl - 1.86 g/L
Buffer A + 0.050 M KCl - 3.72 g/L
Buffer A + 0.5 M KCl - 37.28 g/L

Buffer B + 0.5 M KCl - 37.28 g/L
Buffer B+ 0.2 M KCl- 14.91 g/L
Figure A1-5. **Heparin agarose chromatogram.**

Chromatogram showing the elution of MutSΔ800 protein (mls of gradient eluent) as a function of absorbance at 280 nm.
Figure A1-6. **Elution of MutSΔ800 from Heparin Agarose Column.**

MutSΔ800 protein precipitated from the lysis steps was loaded onto a 30 ml heparin agarose column equilibrated with 50 mM KCl in Buffer A. An aliquot of 10 ul was taken from various fractions according to the chromatogram in Figure 1 to monitor the elution of MutSΔ800 from the column and is shown in the gels below. MutS elutes in fractions B-E, which were pooled for further purification. M-marker, X-remaining wash fractions collected in 50 ml conical tubes.
Figure A1-7. **CHT chromatogram.**

Chromatogram showing the elution of MutSΔ800 protein (mls of gradient eluent) as a function of absorbance at 280 nm.
Figure A1-8. **Elution of MutSΔ800 from CHT column.**

MutSΔ800 protein precipitated from the heparin agarose column was loaded onto a 60 ml CHT equilibrated with 0.2 M KCl in buffer B. An aliquot of 10 ul was taken from various fractions according to the chromatogram in Figure 8 to monitor the elution of MutSΔ800 from the column and is shown in the gels below. MutS elutes in fractions D-E, which were pooled and concentrated. M-marker.
Figure A1-9. **Purity of MutSΔ800 protein.**

The gel shows 10 ul of concentrated protein from pooling fractions D5-E10 (lane 1) and A9-D1 (lane 2) from the CHT column purification. The protein is ~95% pure. Only the D5-E10 concentrate was aliquoted and frozen for use. Exact concentration of the protein was determined using a ninhydrin protein assay. M- marker.
APPENDIX II

REGULATED EXPRESSION OF THE **ESCHERICHIA COLI DAM** GENE

Abstract

Regulated expression of the *E. coli* dam gene has been achieved using the *araBAD* promoter lacking a ribosome binding site. Cultures of dam mutants containing plasmid pMQ430 show no detectable methylation in the absence of arabinose and complete methylation in its presence. Dam methyltransferase is a substrate for the Lon protease.

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