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Assessing the contributions of Dcm and Vsr to spontaneous and chemically-induced DNA damage in *Escherichia coli*

Photini Pitsikas

A Thesis

in

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ABSTRACT

Assessing the contributions of Dcm and Vsr to spontaneous and chemically-induced DNA damage in *Escherichia coli*

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Concordia University, 2002

DNA repair processes are crucial in maintaining genome integrity. Postreplication repair systems target specific misincorporations which occur during DNA replication. The mismatch repair system corrects most base pair mismatches, the nucleotide excision repair system corrects bulky lesions and the very short patch repair system of *E.coli*, repairs G-T mismatches that occur in the CCWGG sequence context.

The purpose of this study is to investigate the roles of very short patch repair, mismatch repair and nucleotide excision repair systems, and their interactions in the presence of mutagen induced lesions caused by 2-aminopurine and 5-azacytidine.

We have examined the regulation of the *dcm* and *vsr* genes of the very short patch repair system, and have found that Vsr is growth phase regulated while Dcm levels remain constant throughout the various stages of the cell's growth. The Vsr regulation was also found to be post-transcriptional and also, although to a lesser extent, operon structure reguiated. This helps explain why 5-methylcytosines are hotspots for mutations in *E.coli*.

We have shown that Dcm, the only cytosine methyltransferase in *E.coli*, when bound to its target site CCWGG and in the presence of mutagens, 2-aminopurine and 5-azacytidine, affects DNA repair and replication, respectively. Our results indicate that

the 2-aminopurine effect is due to increased mispairing of 5-methycytosines with 2-aminopurine compared to normal cytosine.

5-azacytidine stimulates C-to-G mutations which are independent of the methylase. We have evidence which suggests that the lesions leading to the C-to-G mutations are C-C mismatches. We also have results which indicate that these 5-azacytidine induced lesions are repaired by the nucleotide repair system as well as the MutS and MutL proteins of the mismatch repair system. Other lesions caused by the drug include a methylase dependent lesion. Dcm induced lesions occur when the cytosine methyltransferase binds covalently to 5-azacytosine containing DNA. Our results show that this lesion leads to a reduction in DNA replication, cell growth and cell division.

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CONTRIBUTION OF AUTHORS

All the data presented in this thesis is the work of the author, except for the following:

- The growth phase regulation data and preparation of the rabbit polyclonal antibodies, Figure 1 in *Macintyre et al*, 1999.
- pDV105 and pDV106 made by Claire G. Cupples.
- pT18H,L, S and pT25H.L.S made by Catherine Mansour and published in *Mutation Research* 485: 331-8, 2001.
- pMQ348, pMQ339, and pMQ341 made by Martin Marinus, published in *Journal of Bacteriology* 176: 5393-00, 2001.
- The samples for Figure X were prepared by Kathy Doiron and published in *Mutation Research* 429: 37-44, 1999.

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LIST OF ABBREVIATIONS

A: Adenine

5-AC: 5-azacytidine

2-AP: 2-aminopurine

β-gal: β-galactosidase

C: Cytosine

DIG: digoxigenin

2-D: two dimensional

DNA: deoxyribonucleic acid

E.coli: *Escherichia coli*

G: Guanine

Lac: Lactose

LB: Luria Bertani

MMR: Mismatch repair

NER: Nucleotide excision repair

PCR: Polymerase chain reaction

SAM: S-adenosylmethionine

SDS: Sodium dodecylsulfate

T: Thymidine

UV: Ultraviolet

X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactoside

1. INTRODUCTION

DNA repair is a crucial process in maintaining genome integrity. Replication errors during DNA replication occur with a frequency of 10^{-1} to 10^{-2} base pair per replication round. Many DNA polymerases have proofreading exonuclease activity which ensures a low mutation rate. Without the proofreading activity mutation rates would be as high as 1 error in every 10,000 bases (Friedberg *et al.*, 1995). Post replication DNA repair systems recognize mispairs, insertion/deletions, and some other types of DNA alterations that still persist after proofreading. Prokaryotes and eukaryotes share some similar repair systems although they differ with regards to their complexities. The work reported in this thesis addresses the role and regulation of three repair systems of *Escherichia coli* as well as the identification and repair of mutagen induced lesions.

1.1. Mismatch repair

Mismatch repair (MMR) recognizes single base mispairs as well as insertions and deletions that could lead to base substitution and frameshift mutations, respectively (Lahue and Modrich, 1988). MutH, MutL, and MutS make up the three main components of the initiation of the mismatch repair system for the prokaryote *E. coli*. Although the actual mechanism of this repair process still remains unclear, certain aspects have been explored with much detail.

The MutS protein has been shown to recognize and bind to the mispair (Au *et al.*, 1992). In the absence of ATP MutS still binds to the mismatches, however, the ATPase is required for repair (Haber and Walker, 1991). A model proposed involves the binding of MutS to a mismatch followed by subsequent ATP-dependent translocation of one or

more of the Mut proteins along the helix leading to the cleavage of a GATC sequence (Modrich, 1991). There is a hierarchy for the substrates MutS will bind and repair, G/T being the best substrate and C/C being the worst (Lahue *et al.*, 1989;Su *et al.*, 1988;Wagner *et al.*, 1995). Other studies interested in the specificity of mismatch repair performed experiments *in vivo* and used heteroduplex substrates containing different mismatches (Jones *et al.*, 1987;Kramer *et al.*, 1989). The results showed that the efficiency of repair was influenced to some extent by the base composition of the sequence surrounding the mismatch. As in the *in vitro* studies, some mismatches were found to be repaired more efficiently than others. Specifically, the G/T and A/C mismatches, which give rise to transition mutations, are the most efficiently repaired. Of mismatches that give rise to transversion mutations, G/G and A/A mismatches are usually corrected efficiently, some T/T, C/T, and G/A mismatches are corrected less efficiently, while C/C mismatches appear to be subject to very little, if any, methyl-directed MMR (Friedberg *et al.*, 1995). However, a complete mechanism of the system has not yet been established since both MutL and MutS have ATPases and this makes elucidating the different protein functions a challenge.

The MutL protein acts as a "molecular matchmaker" and binds to the MutS-DNA heteroduplex complex. Molecular matchmakers are a class of proteins that use the energy released from the hydrolysis of ATP to cause a conformational change in one or both components of a DNA binding protein to promote the formation of a metastable DNA-protein complex. After the matchmaking the molecular matchmaker dissociates from the complex. This dissociation allows the matched protein to engage in other protein-protein interactions so as to complete its function (Sancar and Hearst, 1993).

E.coli cells methylate their adenines at GATC sites, however, newly synthesized strands are undermethylated. This allows for strand discrimination (Wagner *et al.*, 1984). Evidence for this hypothesis as a preferred model for strand discrimination is the fact that methylation of DNA by sequence-specific methylases lags somewhat behind DNA replication (Lyons and Schendel, 1984; Marinus, 1976). The MutH endonuclease recognizes the hemimethylated sequence and cleaves the newly synthesized unmethylated strand. Following the nick made by MutH, one of four exonucleases, exonucleaseI, exonucleaseVII, RecJ (Cooper *et al.*, 1993), or exonucleaseX (Burdett *et al.*, 2001) removes bases including the mismatched base. DNA polymerase III holoenzyme, helicase II (UvrD), and DNA ligase contribute to finish the repair (Lahue *et al.*, 1989).

Eukaryotic systems such as yeast and humans also posses a mismatch repair system, not unlike that of *E.coli*. However, the mismatch repair processes of these organisms are more complex and involve multiple homologues of the MutS and MutL proteins. A deficiency in the mismatch repair system in mammalian cells, specifically in the *hMSH2* and *hMLH1* genes, has been linked to a predisposition to hereditary nonpolyposis colon cancer (HNPCC) (Fishel *et al.*, 1994;Leach *et al.*, 1993) (Papadopoulos *et al.*, 1994). Since this observation, mutations in other mismatch repair genes, such as hPMS1, hPMS2, hMSH3, and hMSH6, have also been linked to HNPCC (Fishel, 2001; Nicolaides *et al.*, 1994). Table 1 shows the corresponding *E.coli* homologues of these mammalian proteins (Sancar, 1999). *E.coli* has often acted, and continues to act, as a good genetic model system for analyzing mismatch repair, although eukaryotes do not seem to contain a MutH homologue.

Not much is known about strand discrimination in eukaryotes although recent studies have shown that DNA repair functions independently of methylation status at CpG sites (Friedberg *et al.*, 1995). Therefore, these organisms must carry out strand discrimination via another mechanism. Although the information about strand discrimination is limited for eukaryotes, there is evidence for the polymerase being involved in mispair recognition (Flores-Rozas *et al.*, 2000). The DNA polymerase subunit PCNA of *Saccharomyces cerevisiae* has been shown to interact with the repair protein MSH2/MSH6 complex as well as with the PMS1/MLH1 complex, which are homologes in yeast of MutS and MutL, respectively (Clark *et al.*, 2000). Other interactions also have been observed between mismatch repair proteins both in *Saccharomyces cerevisiae* and *Escherichia coli* with the yeast two-hybrid and bacterial two-hybrid systems, respectively (Bertrand *et al.*, 1998) (Mansour *et al.*, 2001). Furthermore, the MutL and MutS proteins, or homologues, have been found to be involved in other repair processes. These include transcription coupled repair in *Escherichia coli* and humans (Mellon *et al.*, 1996; Mellon and Champe, 1996). Transcription coupled repair is a coupling of transcription and nucleotide excision repair. It was demontrated that in actively transcribed DNA, nucleotide excision repair has a pronounced bias for the transcribed strand (Mellon and Champe, 1996).

1.2. Nucleotide excision repair

The repair process known as nucleotide excision repair (NER) is involved in repairing bulky lesions. These include thymine dimers and 6-4 photoproducts which are created by ultraviolet irradiation (reviewed in Sancar and Tang, 1993). In *Escherichia*

coli the initiation of nucleotide excision repair involves three components, the UvrA, UvrB and UvrC proteins. The UvrA protein is commonly referred to as a molecular matchmaker. The homodimer form of this protein binds to the UvrB endonuclease to form a trimer complex. In the present model the UvrA protein loads UvrB onto the DNA at the site of the bulky lesion. Subsequently, this protein-DNA complex induces a conformational change in the DNA which is the substrate for the UvrC endonuclease. Once the UvrC is bound, the UvrB protein nicks the DNA four nucleotides, 3' of the lesion. UvrC then nicks the DNA seven nucleotides, 5' of the lesion. The patch is about 12-27 nucleotides. *E.coli* DNA helicase II (UvrD protein) is required for the release of the resulting oligonucleotide fragment (excision) and for the displacement of UvrC protein. UvrB protein remains bound to the gapped DNA during the excision reaction and is released during the repair sythesis reaction catalyzed by DNA polymerase I. DNA ligation completes the nucleotide excision repair reaction (Sancar and Sancar, 1988).

Nucleotide excision repair in eukaryotic systems is more complex. It contains many more proteins (summarized in Table 1). In mammalian nucleotide excision repair the genes involved were isolated by phenotypic complememtation of DNA-damage-sensitive repair-defective mutants, much like the experiments to isolate the genes involved in the bacterial and yeast systems. One of the mammalian cell systems that has been extensively investigated is the human system. Isolated genes for the human nucleotide excision repair system were derived from three repair-defective human hereditary diseases: xeroderma pigmentosum (XP), Cockayne syndrome (CS), and a

TABLE 1. DNA repair proteins in *E.coli* and humans

E.coli	Human
Excision repair proteins	**Excision repair proteins**
UvrA	XPA
UvrB	XPB (ERCC3)
UvrC	XPC
	XPD (ERCC2)
	XPF (ERCC4)
	XPG (ERCC5)
	ERCC1
	RPA
Mismatch repair proteins	**Mismatch repair proteins**
MutH	–
MutL	MLH1
	PMS1
	PMS2
MutS	MSH2
	MSH3
	MSH6

photosensitive form of trichothiodystropy (TTD) (Broughton *et al.*, 1994; Houtsmuller *et al.*, 1999).

Nucleotide excision repair is not the only repair system in which proteins have been shown to interact with mismatch repair. *E.coli's* very short patch repair system has recently been linked to MMR (Macintyre *et al.*, 1997; Mansour *et al.*, 2001).

1.3. Very short patch repair

The *vsr* gene is located at 43 minutes and overlaps in a +1 reading frame with the *dcm* gene (Bhagwat *et al.*, 1986) that codes for *E.coli* K-12's only cytosine methyltransferase (Figure 1).

Dcm methylates the second cytosine in the CC(A/T)GG sequence. Methylated cytosines can spontaneously deaminate to form thymines. The T/G mismatches are recognized specifically by the Vsr endonuclease (Figure 2). Vsr is a single-stranded endonuclease which cleaves 5' of the mismatched T. Removal of several bases 3' of the nick and their resynthesis by DNA ploymerase I complete the repair process (VSP repair reviewed in Lieb and Bhagwat, 1996). However, even though *E.coli* K-12 has an active *vsr* gene, methylcytosines are still hotspots for mutations (Duncan and Miller, 1980).

Figure 1. Operon organization of *dcm* and *vsr* genes

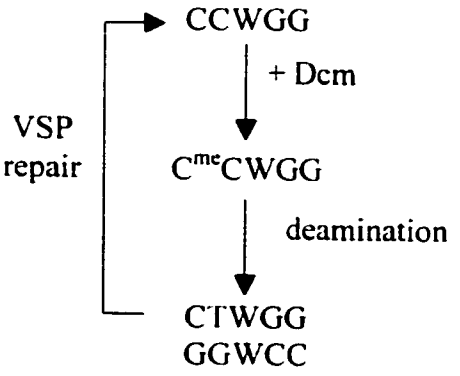


Figure 2. Spontaneous methylated cytosine deamination and VSP repair (W = A or T, ^{me} = methylated)

In addition, when the *vsr* gene is overexpressed this leads to an increase in frameshift and transition mutations (Doiron *et al.*, 1996). The increase in mutations is thought to be due to saturation of the mismatch repair system. The saturation mechanism theory is consistent with the results of genetic assays which show that when more of the MutH and MutL proteins are added to *E.coli* cells over-expressing *vsr*, the Vsr-induced mutagenesis is greatly reduced (Macintyre *et al.*, 1997). Two-hybrid data have confirmed the interaction between Vsr and MutL proteins (Mansour *et al.*, 2001).

As previously mentioned, T/G mispairs are the best substrates for the mismtach repair system and, in general, there is no sequence context needed for recognition or repair, therefore, Vsr and MutS proteins may compete for the same T/G substrate. For this reason, having too much Vsr can bias the repair route of the T/G mispair, leading to TA to CG transition mutations as shown in Figure 3. Also, cytosine methyltransferases have been shown to recognize the T/G heteroduplex making the competition potentially even more complex. Examples of such methlytransferases include M.*Hha*I, M. *Hpa*II, (Yang *et al.*, 1995) and *Eco*RII (Sheluho *et al.*, 1997) and could include Dcm since cytosine methyltransferases are very similar (Kumar *et al.*, 1994). The ability of DNA methyltransferase to block repair of premutagenic lesions, like mispairs, was examined previously. Results included *in vitro* studies that showed blockage of uracil DNA glycosylase by M.*Hha*I (Yang *et al.*, 1995) , as well as *in vivo* studies demonstrating blockage of uracil DNA glycosylase, this time by the M.*Hpa*II methyltransferase

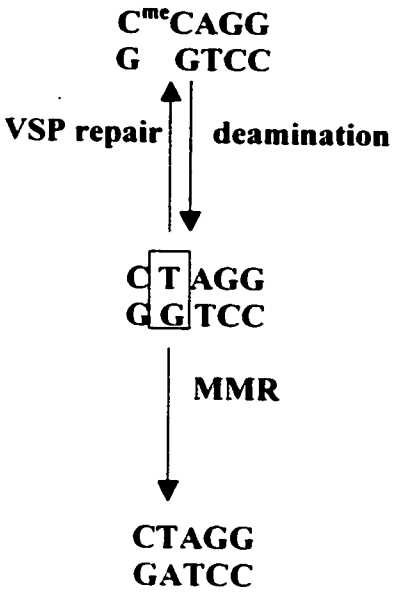


Figure 3: Competition for T/G mismatch in CTAGG sites
This diagram shows how both Vsr and MMR can compete for the same substrate (^{me}=**5-methyl**)

(Yang *et al.*, 1995). Also, it was shown that the blockage of repair was not a general property of methyltransferases and required the presence of the cognate sequence (Yang *et al.*, 1995).

1.4. Methylation

Thirty percent of all human point mutations occur at methycytosines even though 5-methylcytosines are relatively uncommon except when found in concentrated areas such as CpG islands (Jones *et al.*, 1992). Most of the mutations arise from deamination of methylcytosines to thymines (Lutsenko and Bhagwat, 1999). Despite the existence of repair systems dedicated to repairing deamination-induced T/G mismatches, these lesions still persist. Also, 5-methylcytosines are hotspots for C-to-T mutations induced by exogenous mutagens and carcinogens (Coulondre *et al.*, 1978a). In *E.coli*, the repair of the T/G mismatches is highly efficient even though Vsr is found at low levels in dividing cells (Macintyre *et al*, 1997). In humans, the protein responsible for removing the thymine which results from the hydrolytic deamination of 5-methylcytosine is the thymine-DNA glycosylase (Waters and Swann, 2000).

Certain mutagens can serve as important tools to investigate methylation and repair. These include 2-aminopurine and 5-azacytidine.

1.5. 2-aminopurine

2-aminopurine is a base analog which resembles adenine but can also base pair, although less frequently, opposite a cytosine (Ronen, 1980). This mispair with cytosine leads to a 2-AP-C lesion. Incorporation followed by mispairing as a guanine results in an

AT to GC transition, whereas incorporation opposite a cytosine followed by pairing as an adenine leads to a GC to AT transition (Figure 4). Therefore, the formation of 2-AP-C mispairs is the molecular basis for 2-aminopurine mutagenesis. Furthermore, these transition mutations have been shown to occur at three CCAGG sites in the *lacI* gene. These CCAGG sites were found to be hotspots for 2-aminopurine stimulated mutations although the mechanism is still unknown.

In addition, 2-aminopurine also causes frameshift and transition mutations in much the same way as overexpressing *vsr* does, by saturating mismatch repair proteins (Macintyre *et al.*, 1997) since the A/C lesion is recognized by the mismatch repair system (Su *et al.*, 1988). Evidence for the ability of MMR to repair 2-AP-C lesions include *dam* mutant studies. The gene which codes for the DNA adenine methyltransferase, *dam*, is responsible for adenine methylation and strand discrimination for the MMR system. These mutants are very sensitive to the base pair analog 2-aminopurine (Grafstrom *et al.*, 1988). Therefore, MMR can, in fact, recognize these 2-AP lesions that in the absence of strand discrimination can lead to double stranded breaks which cause the *dam* cells to be hypersensitive to the drug (Glickman *et al.*, 1978). Also *mutH* strains suppress the lethal effect of 2-AP in *dam* strains further supporting the theory that MMR can repair 2-AP-C lesions (Glickman and Radman, 1980).

Consequently, the balance of all repair proteins in the presence of these and other similar lesions can be altered, especially in certain sequence contexts. Also, the presence of methylases that recognize these sites leads to a competition between repair systems for the same substrate. This is one of the main issues addressed in this thesis.

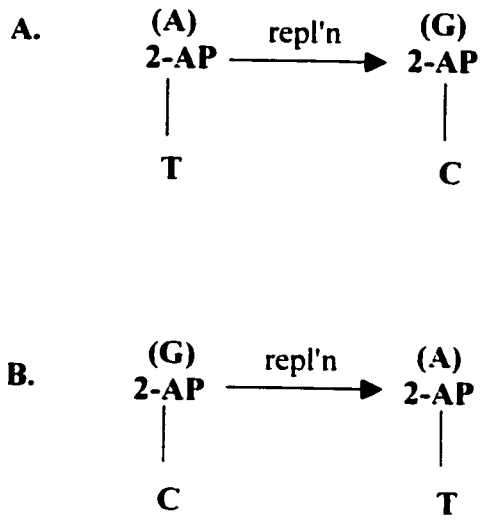


Figure 4: **A.** 2-AP base analog base paired opposite a thymine followed by mispairing opposite a cytosine leads to a AT to GC mutations. **B.** 2-AP incorporated opposite a cytosine followed by pairing opposite a thymine leads to GC to AT mutations.

1.6. 5-azacytidine

5-azacytidine is a nucleoside analog that was originally synthesized as a chemotherapeutic agent. It has been used in the treatment of human myelogenous leukemia. It works as a demethylating agent to activate genes which have been erroneously silenced or down-regulated through methylation. Much work has been done to understand the role of DNA methylation in gene expression. Therefore, drug induced DNA hypomethylation has up until now proven to be a very powerful tool in understanding eukaryotic gene expression. Despite the fact that 5-azacytidine is nonspecific, it activates genes in a selective manner and does not cause a global increase of gene expression (Jones, 1985). Genes that are activated include the thymidine kinase gene (Jones, 1985) and the p73 supressor gene (Kawano *et al.*, 1999). 5-azacytidine is also used in analysis of development in eukaryotic systems by inducing cellular differentiation (Jones and Taylor, 1980). Examples include the development of contractile striated muscle cells, and biochemical differentiation of adipocytes and chondrocytes (Jones and Taylor, 1980). Another important discovery involving the effect of 5-azacytidine is that certain analogs of 5-AC lead to cellular differentiation and inhibition of DNA methylation. These analogs contained cytidines which were altered at the 5-position. Analogs which did not lead to the same desired effects had alterations at positions other than the 5^{th} carbon. Consequently, 5-azacytidine's mechanism of action seems to involve the C5-postion of the pyrimidine ring (Jones and Taylor, 1980).

Recently, the analog has been shown to cause C-to-G transversion mutations both in mammalian cells (Kelecsenyi *et al.*, 2000) and in *Escherichia coli* (Cupples and Miller, 1989). Although most mutations noted in mammalian systems were C-to-Gs,

some C-to-Ts and C-to-As occurred at a lower frequency which differs from the effect seen in *E.coli* cells (Jackson-Grusby *et al.*, 1997). The C-to-G mutations have been shown to be dose-dependent (Cupples and Miller, 1989). There is, however, disagreement over whether or not the methylase is involved in the 5-AC induced mutagenesis.

1.6.1 Dcm independent lesions

In *E.coli*, 5-azacytidine-induced C-to-G mutations (Doiron *et al.*, 1999) occur at methylatable C's as well as non-methylatable C's and these mutations occur whether the Dcm methylase is present or not. C-to-T mutations occur in *E.coli* when the Dcm methylase is present in the absence of the *vsr* gene. As the concentration of 5-azacytidine increases, the number of C-to-T mutations decreases while the number of C-to-G mutations increases. Therefore, Dcm does not stimulate, nor does it reduce, the C-to-G transversion mutations caused by 5-azacytidine. Also, since increased levels of the drug decrease the C-to-T transition mutations in a *dcm*$^-$ *vsr*$^-$ strain, 5-azacytosines replace 5-methylcytosine which leads to de-methylation of the genome and a clear indication that the drug is being incorporated into the DNA in these studies. Dcm does, however, cause a slight but significant increase in mutations at lower concentrations of 5-azacytidine (Doiron *et al.*, 1999). However, 5-azacytidine-induced C-to-G mutations are independent of the methylase, both in *E. coli* and in mammalian cells (Doiron *et al.*, 1999) (Kelecsenyi *et al.*, 2000). However, an analogue of 5-azacytidine, 5-aza-2'-deoxycytidine, in a similar study of monitoring drug induced mutations, has been shown

to be dependent on the methylase, therefore, there is some ambiguity with regard to the mammalian system (Jackson-Grusby *et al.*, 1997).

The C-to-G mutations have been shown to occur due to the incorporation of the cytidine analog in the DNA as 5-azacytosine (Friedman, 1985) followed by a mispair. The only direct way of producing a C-to-G mutation is through a C-C or G-G mismatch. The G-G mispair is a good substrate for mismatch repair however the C-C lesion is the worst substrate for the system. However, it has since been demonstrated that C/C mispairs are corrected in *Schizosaccharomyces pombe* by the nucleotide excision repair system (Fleck *et al.*, 1999;Sancar, 1999).

Furthermore, interactions between the MSH2 (MutS homolog, refer to Table 1) protein of the mismatch repair system and many of the nucleotide excision repair proteins (including Rad1, Rad2, Rad3, Rad 10, Rad14 and Rad25, which have similar activities to the UvrA and UvrB proteins in the *E.coli* NER system) have been shown in *Saccharomyces cervisiae* using the yeast two-hybrid system and coimmunoprecipitations (Bertrand *et al.*, 1998). This finding was consistent with the earlier publications implicating the MutS and MutL proteins, and their homologues in humans, in transcription-coupled excision repair in *E.coli* and mammalian cells (Mellon *et al.*, 1996;Mellon and Champe, 1996). Mismatch repair proteins also have been shown to interact with the very short patch repair proteins of *E.coli* using the yeast two-hybrid system, as well as the bacterial two-hybrid system (Mansour *et al.*, 2001) which has proven to be more efficient than the yeast system for looking at interactions between bacterial proteins (Mansour *et al.*, 2001).

There is little known about the 5-azacytidine induced lesion which causes the C-to-G mutations or how it is repaired.

1.6.2. Dcm dependent lesions

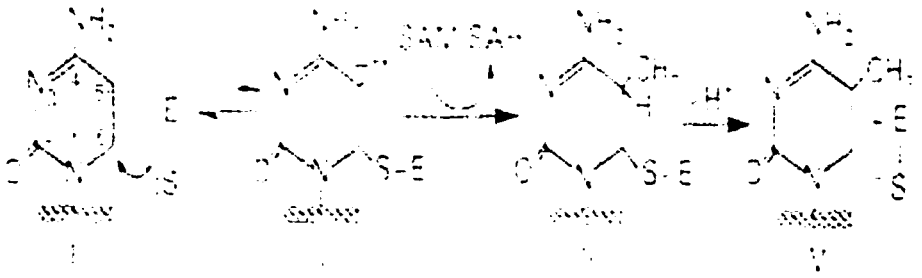
5-azacytidine also has been shown to be mutagenic in a variety of cellular systems. It is also toxic, especially in cells that overexpress a DNA cytosine methyltransferase (Juttermann *et al.*, 1994). The effect was demonstrated to be dependent on the amount of the methyltransferase present in the cell. This effect was also seen with the methyltransferases Dcm and *Msp*I (Friedman, 1982). The mechanism of the drug induced cytotoxicity is not well understood. It was postulated that the toxicity of the drug in mammalian cells was due to its inhibition of DNA methylation, and although the methylation of cytosines in mammalian systems has been linked to cancers, studying 5-azacytidine's effects in animal systems devoid of cytosine methylase is unfeasible due to the fact that this would lead to embroyonic lethality (Li *et al.*, 1992). However, prokaryotic systems such as *Escherichia coli* serve as a good model for such studies since *E.coli* cells devoid of their only cytosine methylase, Dcm, are viable. The hypothesis for the drug causing toxicity through hypomethylation was highly plausible. However, an alternative mechanism involves 5-azacytosine-containing DNA covalently bound to the methyltransferase leading to blockage in DNA replication.

In vitro studies with 5-azacytidine have shown that the methyltransferase actually remains trapped in a covalent complex with DNA containing 5-azacytosine. These DNA-cytosine methyltransferases include the *Eco*RII, *Msp*I, and Dcm methylases (Friedman, 1985). The complexes are very stable and could not be dissociated with high

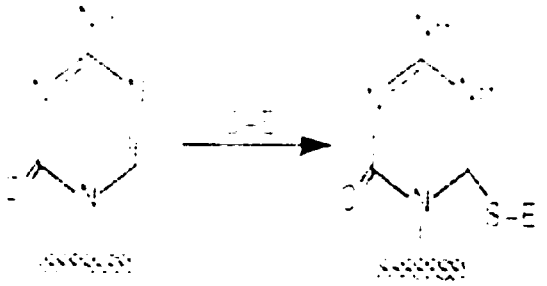
salt concentrations or detergents. Also, the complex formation is stimulated when S-adenosylmethionine is added, and the binding is dependent upon the presence of both the methylase recognition sequence and the azacytosine in that DNA (Friedman, 1985).

The mechanism of inhibition is proposed to include the DNA cytosine 5-methyltransferase catalyzing an electrophilic subsitution at the fifth position of the targeted cytosine which is believed to undergo the SAM-dependent mechanism of methylation shown in Figure 5a. The cysteine residue of the catalytic center of the DNA cytosine 5-methyltransferase undergoes Michael addition to the C6/C5 double bond of the target cytosine residue activating the C5 atom as a nucleophilic center. Methyl transfer is followed by the regeneration of the catalyst via β-elimination. When 5-azacytosine is the base instead of cytosine (Figure 5b) then the β-elimination step would be blocked, the methyl transfer could not take place, and the covalent structure would be irreversible (Gabbara and Bhagwat, 1995). Little has been done to show how this lesion is repaired but the cell's continued viability with relatively high doses of 5-azacytidine seems to indicate that it must be repaired somehow.

Figure 5. Mechanism of inhibition



a) Mechanism of cytosine methylation



b) The SAM-independent mechanism of the inhibition of C5 Mtase by 5-azacytosine

Some research has been done to determine which repair systems are involved in the correction of the 5-azacytidine-induced damage. The work included testing various repair deficient mutants for sensitivity to the drug. It was postulated that the cytosine methyltransferase-5-AC complex apparently was removed since a *dcm*$^+$ *E.coli* strain was only slightly more sensitive than a *dcm*$^-$ strain. Recombinational repair and nucleotide excision repair are the two most likely systems for targeting the bulky lesion. *E.coli* mutants that were most sensitive to the toxicity of the drug were in fact strains deficient in recombinational repair genes including *recA* and *lexA* (Bhagwat and Roberts, 1987) (Cupples, unpublished results). Also, *uvrA* and *mutS* mutant strains of the nucleotide exision and mismatch repair system, respectively, were also sensitive (Hegde *et al.*, 1996). The mismatch repair protein MutS has been shown to be involved in recombinational repair and more recently in transcription coupled repair (Mellon and Champe, 1996). Therefore, the results suggested that sensitivity of the *mutS* homolog deficient strain could be due to participation of the protein in repair of the lesion.

In addition, 5-azacytidine has been shown to cause a weak induction of the SOS response (Barbe *et al.*, 1986). The SOS response is a system which induces transcription of DNA repair genes in response to DNA damage (reviewed in Little and Mount, 1982). The genes involved in the SOS response also include recombinational repair genes, *recA* and *lexA*, two genes which are induced by the SOS response. Put together, these results strengthen the likelihood that recombinational repair and nucleotide excision repair are involved in the repair of the 5-azacytidine-induced lesions.

1.7. Thesis Overview

We have summarized three main repair systems of *E.coli* which will be examined in one way or another in this study. Next, methylation which is important in studying repair systems for many reasons (one of which is that it can indirectly cause mispaired bases which are substrates for VSP and MMR) was briefly reviewed. Another reason, is that the cytosine methylase can contribute to a lesion which may block repair or block the replication machinery when DNA contains base analogs such as 2-aminopurine and 5-azacytosine. To study the repair of deamination-induced T/G mispairs and methylase dependent lesions, two mutagens, 2-aminopurine and 5-azacytidine were used and, therefore, described in the last part of this introduction.

The results of this thesis are separated into four subsections. The regulation of the Vsr and Dcm genes comprise the first section (3.1). The low levels of Vsr protein during *E.coli's* growth phase (Macintyre *et al.*, 1999) together with previously-reported information that interactions exist between repair systems brings us to sections 3.2, 3.3 and 3.4, where the mutagens 2-aminopurine and 5-azacytidine are used to investigate these overlaps. The role of the methylase on recognition of sequence specific premutagenic mispairs, and the resulting lesions, will also be examined in sections 3.2 and 3.4. Finally, 5-azacytidine also has been shown, as previously mentioned, to cause mutations that are independent of the cytosine methylase and the interactions between the various repair systems in repairing these 5-AC lesions is presented in section 3.3.

2. MATERIALS AND METHODS

2.1. *Escherichia coli* transformations

Plasmid DNAs were introduced into *E.coli* K-12 cells by the calcium chloride technique (Sambrook *et al.*, 1989). Transformants were selected on either Luria-Bertani or minimal glucose medium containing the appropriate antibiotics. The final concentrations of antibiotics used were as follows: Ampicillin, 100μg/ml, chloramphenicol, 20μg/ml, kanamycin, 50μg/ml, streptomycin, 100μg/ml, and tetracycline, 15μg/ml.

2.2. Western blot analysis

E.coli cell extracts containing equal amounts of protein were electrophoresed on sodium dodecyl sulfate-polyacrylamide (SDS) gels, and the separated proteins were transferred to PVDF transfer membranes (NEN™ Life Science Products) for Western analysis. The proteins were detected using Dcm and Vsr primary rabbit antibodies, followed by goat anti-rabbit secondary antibody, which was conjugated to HRP (horse radish peroxidase). The chemiluminescent substrate, Luminol was used. The procedure used was outlined by the supplier (NEN™ Life Science Products).

2.3. *E.coli* strain construction

Bacterial strains used for the 5-azacytidine mutagenesis experiments were CC103 and CC197 which are derivatives of CSH142 and contain an episome F' *lacZYA, proAB* (refer to Table 2 for strain descriptions). The strains differ only in the nature of the

mutation in the *lacZ* gene. The CC103 episome reverts from Lac^- to Lac^+ by the C→G transversion mutation at codon 461 and the CC107 strain by a + G frameshift.

Mismatch repair deficient strains were made via P1 transductions of *mutS201*::*Tn5* , *mutL211*::*Tn5*, and *mutH471*::*Tn5* into CC103 and CC112 (described below) recipient strains. All transductants were selected for kanamycin resistance. Mutator phenotypes for the CC103*mut* strains were checked by spotting 10 μl of the saturated cultures onto LB medium containing rifampacin (100 μg/ml).

Nucleotide excision repair deficient strains were also made via P1vir transduction. P1vir lysates were made using strains: PF101, CAG12156, and N3055 for introduction into CC103. The *uvr* phenotype was checked by plating cells onto LB media for viability and treating them with increasing doses of ultraviolet light. During and after exposure to UV light, plates were kept in the dark to avoid the activation of the photolyase. Mutant cells were more sensitive to the UV light than wildtype cells.

Polymerase IV deficient strain, (BS40Δ*dinB*/F'CC107Δ*dinB*), was obtained from B. Strauss, University of Chicago. The strain contains the F' CC107 episome and, therefore, monitors a frameshift mutation. The strain contains a *dinB* mutation on the chromosome and the episome, making it a complete polymerase IV mutant.

The cytosine methyltransferase deficient strain used for 5-azacytidine-induced frameshift studies is a deletion mutant that also is deleted for the *vsr* gene and other genes located in the deleted area (see genotype). The strain was made by a conjugation of the F' CC107 episome into CC221 strain.

TABLE 2. Strain descriptions

Strain	Genotype	Reference or source
CSH142	*ara* Δ(*gpt-lac*)5 *thi*	Miller 1992
CC103	*ara* Δ(*gpt-lac*)5 *thi* F' *lacZYA*, *proAB*	Cupples *et al*, 1989
CC103*mutH*	*ara* Δ(*gpt-lac*)5 *thi* F' *lacZYA*, *proAB mutH*::*Tn5*	This study
CC103*mutL*	*ara* Δ(*gpt-lac*)5 *thi* F' *lacZYA*, *proAB mutL*::*Tn5*	This study
CC103*mutS*	*ara* Δ(*gpt-lac*)5 *thi* F' *lacZYA*, *proAB mutS*:: *Tn5*	This study
CC103*uvrA*	*ara* Δ(*gpt-lac*)5 *thi* F' *lacZYA*, *proAB uvrA*:: *Tn10*	This study
CC103*uvrB*	*ara* Δ(*gpt-lac*)5 *thi* F' *lacZYA*, *proAB uvrB*:: *Tn10*	This study
CC103*uvrC*	*ara* Δ(*gpt-lac*)5 *thi* F' *lacZYA*, *proAB uvrC*:: *Tn10*	This study
CC107	*ara* Δ(*gpt-lac*)5 *thi* F' *lacZYA*, *proAB*	(Cupples *et al.*, 1990)
CC107Δ	*ara* Δ(*gpt-lac*)5 *thi* F' *lacZYA*, *proAB* Δ(*supD-dcm-fla*) *zee*3129::*Tn*10	This study
BS40Δ*dinB*/F'CC107 Δ*dinB*	*metB* Δ (*proAB lac*) *rpsl* Δ*dinB*::*kan*/F' *lacIZ proB*$^{+}$ Δ*dinB*::*kan*	(Strauss *et al.*, 2000)
CC107*vsr*::*kan*	*ara* Δ(*gpt-lac*)5 *thi* F' *lacZYA*, *proAB vsr*::*kan*	This study
CC112	*ara* Δ(*gpt-lac*)5 *thi gyrA* *argE*(Am) *rpoB* F' *lacZYA*, *proAB*	(Ruiz *et al.*, 1993)
CC112Δ	*ara* Δ(*gpt-lac*)5 *thi gyrA* *argE*(Am) *rpoB*Δ(*supD-dcm-fla*) *zee*3129::*Tn*10 F' *lacZYA*, *proAB*	(Macintyre *et al.*, 1997)
CC113	*ara*Δ (*gpt-lac*)5 *thi*, F' *lacZYA*, *proAB*	(Doiron *et al.*, 1996)
CC113Δ	*ara*Δ (*gpt-lac*)5 *thi* Δ(*supD-dcm-fla*) *zee*3129::*Tn*10, F' *lacZYA*, *proAB* Δ(*supD-dcm-fla*) *zee*3129::*Tn*10	This study
CC221	*ara*Δ(*gpt-lac*)5 *thi gyrA* Δ(*supD-dcm-fla*) *zee*3129::*Tn*10	(Doiron *et al.*, 1996)
N3055	*uvrA277*::*Tn10*	*E.coli* stock center

PF101	*uvrB5, chlA::Tn10*	(Foster *et al.*, 1983)
CAG12156	*uvrC279::Tn10 rph*$^{-1}$	*Ecoli* stock center
LJ2809	*fruR1*::Tn10, *xylA7*, Δ*cyaA854*, Δ*argH1*	*E.coli* Stock center

CC221 was transformed with plasmids containing *dcm* cloned into pACYC184 (Table 3). pDV105 and pDV107 have a C177S mutation and a C177W mutation in *dcm* respectively (Doiron *et al.*, 1999;Wyszynski *et al.*, 1993).

Table 3. pACYC based plasmids

Plasmid	*Dcm*	*vsr*	Reference or source
pACYC184	No	no	New England Biolabs
pDCM28	no	yes	(Sohail *et al.*, 1990)
pDV101	wildtype	no	(Doiron *et al.*, 1996)
pDV102	wildtype	yes	(Doiron *et al.*, 1996)
pDV105	C177S mutant	no	(Doiron *et al.*, 1999)
pDV106	C177S mutant	yes	(Doiron *et al.*, 1999)
pDV107	C177W mutant	no	(Cupples, unpublished)

Strains used for the 2-aminopurine experiments contain episomes for detecting specific C-to-T and T-to-C transition mutations in the *lacZ* gene. CC112 contains a plasmid with a synthetic tRNA gene. The tRNA inserts a glutamic acid at UAG (amber) codons. CC112 monitors the CCAGG to CTAGG change at codon 461 in the *lacZ* gene. CC112Δ also was used to monitor the same change as CC112 but this strain is deficient for the

dcm gene. CC112Δ was also transformed with plasmids, pACYC184, pDV101, pDV102, pDV105, pDV106, pDV107 and pDCM28 (all described in Table 3). CC113 and CC113Δ were transformed with control plasmid pACYC184, and pACYC184 based plasmids containing MMR genes, pMQ348 (*mutH*), pMQ339 (*mutL*), and pMQ341 (*mutS*). The MMR plasmids were obtained from Martin Marinus, University of Massachusetts.

2.4. Lactose reversion assays

To monitor the specific C-to-G mutations that a drug causes, particular *E.coli* Lac^- test strains were used. The Lac^- to Lac^+ phenotypic change in each individual strain can only occur due to a specific mutation. Cultures were diluted 10^{-6} and 100 µl were plated in duplicate on LB agar and 100 µl of the undiluted cultures were spread in duplicate onto minimal lactose plates. Lac^+ revertant colonies were counted after 36-40 hours. Colonies on LB plates for viability after overnight incubation. Numbers of Lac^+ revertants/10^8 viable cells were calculated.

Also, for the 2-aminopurine experiments with the CC112 cells, 10 µl of saturated culture was spotted onto papillation plates. Papillation plates are made by adding 40 µg/ml of X-gal and 500 µg/ml of P-gal in minimal glucose medium plus agar (Miller, 1992).

2.5. Treatment of *E.coli* cells with 2-aminopurine and 5-azacytidine mutagens

For treatment of *E.coli* cells, stock solutions of the drugs were added directly to growth medium. 2-aminopurine was made in LB or minimal glucose medium at a stock

concentration of 700 ug/ml and stored at 4°C (Miller, 1992). 5-aza cytidine was made fresh in minimal glucose medium at a stock concentration of 1mg/ml as described in Cupples and Miller (1989).

2.6. 2-D gel electrophoresis

Replication intermediate DNA was prepared as described by (Krasilnikova *et al.*, 1998). The two dimensional electrophoresis technique was also preformed as published (Krasilnikova *et al.*, 1998) with the following modifications. The first dimension gel was a 0.3% agarose gel made in 1X TBE buffer and the second dimension gel was a 1.2% TBE agarose gel. The probe used was a *Hind*III (MBI fermentas) linearized pACYC184 plasmid. The probe was labeled with dioxigenin (DIG) using the DIG DNA labeling and detection kit from Boehringer Mannheim. The replication intermediate DNA was digested with BssSI from New England Biolabs to yield a 5.8 kilobase linear DNA molecule. DNA was transferred onto a Biotrans^{TM} nylon membrane (ICN) via Southern blot technique outlined in (Sambrook *et al.*, 1989). Detection of the replication fork was performed using alkaline phosphatase as a substrate with the DIG kit (Boehringer Mannheim) and Ambion's ULTRAhyb ultrasensitive hybridization buffer.

2.7. Bacterial two-hybrid assay and media

Bacterial two-hybrid interactions were detected by transforming the various combinations of compatible fusion plasmids into the *E.coli* strain LJ2809, obtained from the *E.coli* Genetic Stock Center. These transformants were grown in LB medium at 30 °C with aeration for between 12-16 hours with various concentrations of 5-azacytidine. The

saturated cultures were then spread (100 µl) and spotted (10 µl) onto an LB agar Petri plate containing 40 µg/ml of X-gal, 100 µg/ml of ampicillin, 30 µg/ml of chlorophenicol, and 15 µg/ml of tetracycline.

2.8.Plasmid DNA analysis

Plasmid DNA was extracted from equal numbers of cells (as determined by OD_{600}) using standard protocols outlined in (Sambrook *et al.*, 1989). Equal volumes of undigested DNA were electrophoresed on a 1% agarose gel and visualized using ethidium bromide and the 312 nm Transilluminator FBTI88 from Fisher Scientific.

2.9. Cell staining and microscopy

The Zeiss Axioplan photomicroscope was used to visualize the heat fixed cells stained with methylene blue. Methylene blue stain was made by mixing 1:3.34 of a solution of 1% methylene blue in 95% ethanol with a solution of 0.1 mg/ml potassium hydroxide.

2.10. Plasmid constructions

Clones of *uvr* genes in the bacterial two hybrid vectors, pT18 and pT25, were made by using PCR primers designed to place the genes in frame with the adenylate cyclase catalytic domain fragments and by engineering restriction sites. The pT18 plasmid is a derivative of pBluescript II KS (Stratagene), and the pT25 plasmid is a derivative of pACYC184. These bacterial two-hybrid plasmids are described in

(Karimova *et al.*, 1998). The sequences of all the synthetic oligonucleotides used in making these clones are shown in Table 4.

TABLE 4. Sequence of the synthetic oligonucleotides used for bacterial two hybrid vectors containing the NER genes

uvrA (pT25/PstI)	5'-GCGTTTAATCCCTGCAGGGTGAATGGA-3'
uvrA (pT25/BamHI)	5'-CTCAGAAAGGATCCTTAACGAT-3'
uvrA (pT18/XhoI)	5'-AAAGCGTTTACTCGAGGAAAGGTGA-3'
uvrA (pT18/HindIII)	5'-CGGCCTTAAGCTTTTCAGCATCGGC-3'
uvrB (pT25/PstI)	5'-TCCTTCAGGTCTGCAGTCATGAGTAA-3'
uvrB (pT25/BamHI)	5'-GTCTTCTTCGGGATCCTGTTAC-3'
uvrB (pT18/XhoI)	5'-CAACTCCTTCTCGAGGCGACTCAT-3'
uvrB (pT18/HindIII)	5'-CAGTCTTCTAAGCTTTCCTGTTTCGATGCCGC-3'
uvrC (pT25/PstI)	5'-GTAACCAGCCCTGCAGGCGTTTA-3'
uvrC (pT25/BamHI)	5'-TACCCCTATGGATCCACAGAGAC -3'
uvrC (pT18/XhoI)	5'- AACCGTAACCCTCGAGCCAGGCGT -3'
uvrC (pT18/HindIII)	5'-TTGCCACAGAAGCTTCTATGTTTCAAC -3'

3. RESULTS

3.1. Defining the regulation of *vsr* and *dcm*

It has been shown that inefficient VSP repair in replicating cells is a major contributor to mutation at 5-methylcytosine in *E.coli* (Lieb and Rehmat, 1997). Recently, we proposed that the inefficiency is a deliberate strategy for mutation avoidance (Macintyre *et al.*, 1997). The counterintuitive hypothesis is based on our finding that artificially increased production of Vsr results in very high levels of mutation at non-CCWGG sequences (Doiron *et al.*, 1996). The types of mutation which occur (transitions and frameshifts), the frequency with which they occur, and the fact that their frequency is reduced by addition of plasmids containing *mutH* and *mutL* (Macintyre *et al.*, 1997) all suggest that Vsr-stimulated mutation is caused by interference with mismatch repair. If this is indeed the case, then Vsr production may have to be maintained at low levels in dividing cells to ensure optimal correction of DNA replication errors. Unfortunately, this strategy could also result in suboptimal VSP repair and a subsequent increase in C-to-T mutations at 5-methylcytosines.

A rabbit polyclonal antibody to Vsr was used to measure production of the protein during the growth of a culture of *E.coli*. Cell extracts containing equal amounts of protein from CSH142 cells at various times throughout the log phase and stationary phase (Figure 6A) were analyzed. Figure 6B shows that Vsr is not detectable during log phase, appearing only as the cells enter stationary phase.

The 5' end of *vsr* overlaps the 3' end of *dcm* in a +1 reading frame (Sohail *et al.*, 1990), and the genes are apparently co-transcribed from a promoter 5' of *dcm* (Dar and Bhagwat, 1993). If the stationary-phase-dependent regulation of Vsr production were

transcriptional, one would expect Dcm production to follow the same pattern as Vsr. Figure 6C shows the results of Western analysis with a polyclonal antibody that was made specific for Dcm. Unlike Vsr, the cellular levels of Dcm are independent of growth phase, strongly suggesting that regulation of Vsr production is post-transcriptional.

3.1.1. Vsr is post-transcriptionally regulated

To confirm that Vsr is post-transcriptionally regulated, we used a plasmid, pKK-DV (Macintyre *et al.*, 1997), in which *dcm* and *vsr* are expressed from a plasmid-borne promoter (trc). Transcription of the operon is constitutive since the strain is *lacI*. Cells containing pKK-DV were collected in early log, late log, and stationary phases and these cell extracts were subjected to Western analysis. Production of Dcm from the plasmid remained independent of growth phase (Figure 7C); production of Vsr remained dependent on growth phase (Figure 7D).

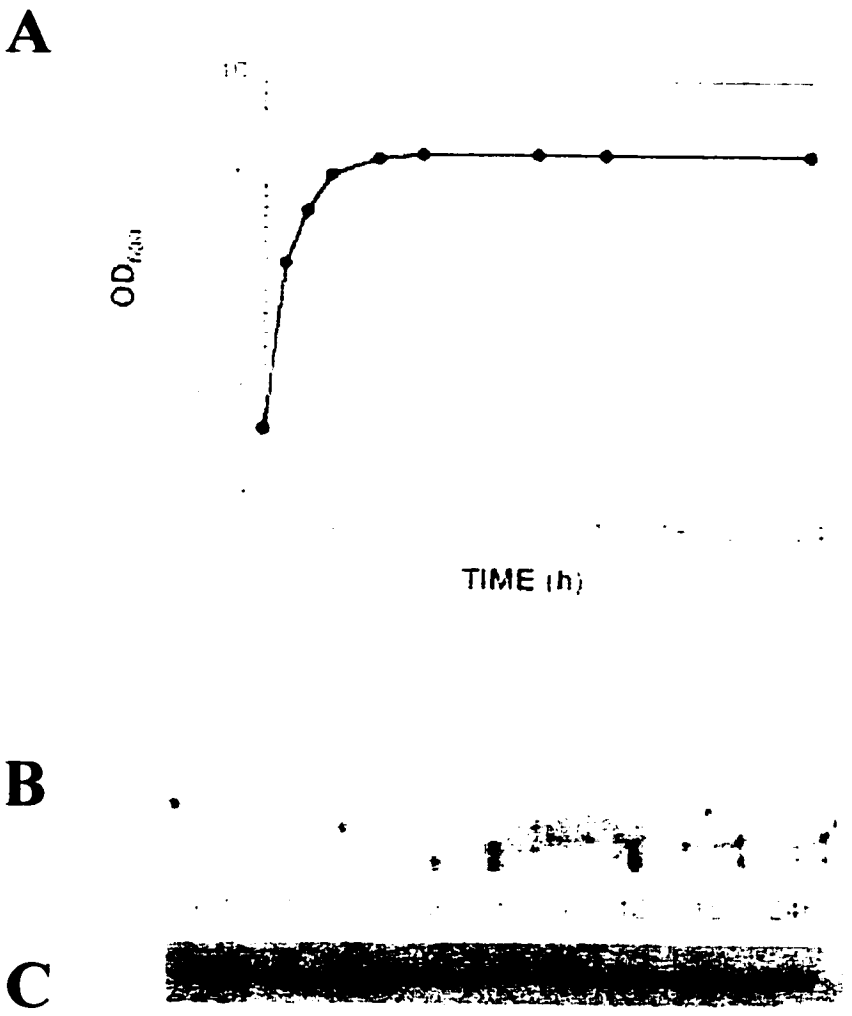


Figure 6. Growth phase regulation of Vsr and Dcm. Production of Vsr and Dcm in growing and stationary phase *E.coli*. Cultures were initiated from 1:100 dilution of an overnight, saturated culture. Culture growth was measured by optical density at 600 nm. (**A**). Samples were taken from the culture at the times shown, and protein extracts were subjected to Western analysis using antibodies to Vsr (**B**) or to Dcm (**C**).

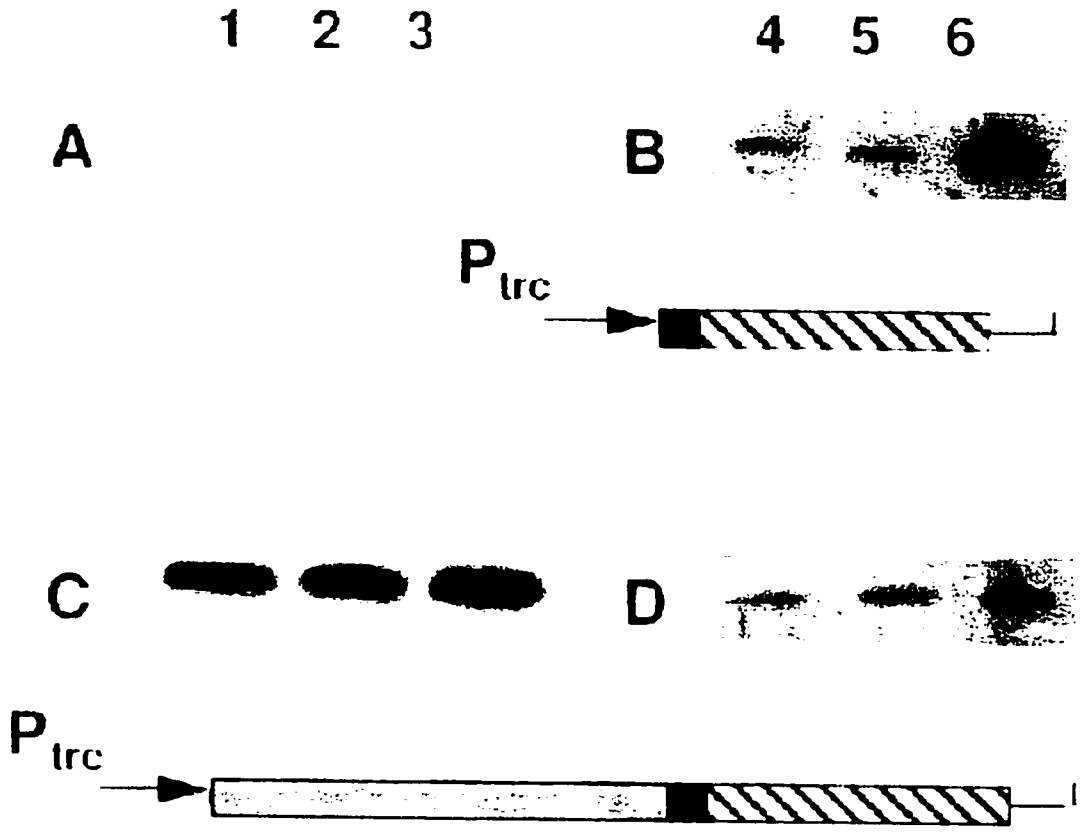


Figure 7: Growth phase-dependent production of Vsr is independent of the *dcm* promoter and of operon structure. Cells were transformed with pKK-V (A,B) or pKK-DV (C,D); maps of the corresponding plasmid inserts are shown. Cultures were grown in LB plus ampicillin from a 1:100 dilution of a saturated overnight culture. Samples were taken in early log (lane 1, 4), late log (lane 2,5) and stationary phase (lane 3, 6), and protein extracts were subjected to Western analysis. The top half of each blot was treated with antibodies to Dcm (A, C) and the bottom half with antibodies to Vsr (B, D). The small amount of Dcm visible in Fig. 7A is the product of the chromosomal *dcm* gene.

3.1.2. Operon structure affects levels of Vsr

Vsr disappears very rapidly when stationary-phase cells are diluted into fresh medium (Figure 6B), suggesting that the protein is actively degraded as cells prepare to re-enter log phase. The subsequent slow buildup of the protein could be due to inefficient translation, due to the fact that the *vsr* ribosome binding site (RBS) is within the 3' end of the *dcm* coding region (Sohail *et al.*, 1990). To determine whether this is the case, we measured Vsr production in cells transformed with pKK-V (Doiron *et al.*, 1996). In this plasmid, *vsr* is transcribed directly from the *trc* promoter and translated from a plasmid-borne RBS. pKK-V transformants produce higher levels of Vsr than pKK-DV in stationary phase (Figure 8, lanes 3 and 4). These higher levels are also noted when different synthetic promoters are used such as the pBAD promoter which is controlled and induced by arabinose concentrations (Figure 8, lanes 1 and 2). These results indicate that there also is operon structure regulation that affects the VSP genes.

3.1.3. Vsr regulation in protease mutant

ClpP is one of the main proteases targeting proteins for proteolysis in *E.coli*. For this reason, strains mutant for this gene were used. *clpP* deficient cells and the wildtype isogenic strain were grown and protein extracts were isolated at mid-log and stationary phases. Western blot analysis was performed and the results are shown in Figure 9. ClpP is not the protease responsible for the post-transcriptional regulation of Vsr since the deficient strain, Lanes 3 and 4, still displays post-transcriptional regulation as can be seen with the low levels of Vsr in Lane 1 and 3.

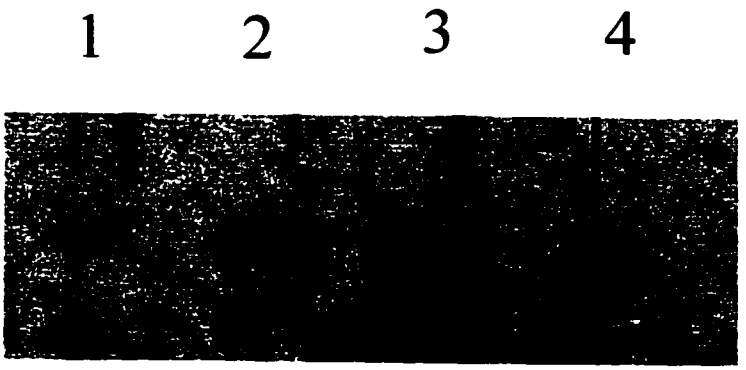


Figure 8. Operon structure regulation. Protein extracts were isolated from CSH142 cells containing plasmids expressing *vsr*. Lanes 1 and 2 show Vsr protein from cells in mid-log and stationary phase respectively. These cells expressed *vsr* from a synthetic promoter (P_{BAD}). Lanes 3 and 4 show Vsr levels from mid-log and stationary phase cells, respectively, and are from cells expressing *vsr* from the P_{TRC} promoter.

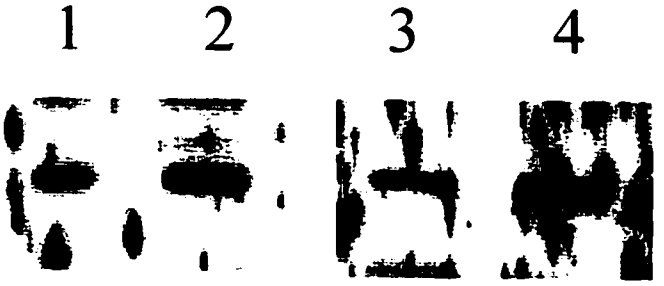


Figure 9. Vsr regulation in protease mutant. Western blot analysis for Vsr shown in lanes 1-4. Lanes 1 and 2 are wildtype CSH142 cells and Lanes 3 and 4 are from *clpP* strain. Lanes 1 and 3 are from cells at mid-log phase, and lanes 2 and 4 are from stationary phase cells.

3.2 5-methylcytosines are hotspots for 2-aminopurine-induced C-to-T mutations

Methylated cytosines are hotspots for spontaneous C-to-T mutations in *E.coli*. These mutations are thought to be due primarily to deamination of 5-methylcytosine to thymine. Methylated cytosines are also hotspots for C-to-T mutations caused by the base analog, 2-aminopurine. 2-aminopurine causes both C-to-T and T-to-C mutations by mispairing with C. To understand why 5-methylcytosines are particularly susceptible to mutation by 2-aminopurine we used two types of cells. Both strains monitor changes which occur at the methylatable site of CCWGG. The CC112 strain monitors CCAGG-to-CTAGG mutations and the CC113 strain monitors CTAGG-to-CCAGG mutations, both at specific sequences in *lacZ* (Figure 10). Those specific mutations are the only ones which will cause the test strains to change the phenotype from Lac^- to Lac^+.

These test strains allow us to address our two main hypotheses. The first, is that the Dcm methylase blocks access of MMR to the 2-AP-C lesion. The second, is that methylcytosines mispair more easily with 2-aminopurine than normal cytosine does. The use of these strains should enable us to determine which of these hypotheses is most feasible. This is possible because CC113 sites are not methylatable whereas CC112 sites are. However AP-C can presumably still be bound by the methylase in both 112 and 113. The latter assumption is based on prior published data which showed that other cytosine methyltransferases can bind heteroduplexes within methylation sites (Yang *et al.*, 1995). Another advantage of using CC112 is that since it is a methylatable site, it can be used to investigate whether methylcytosines base pair more frequently with 2-aminopurine base than does regular cytosine.

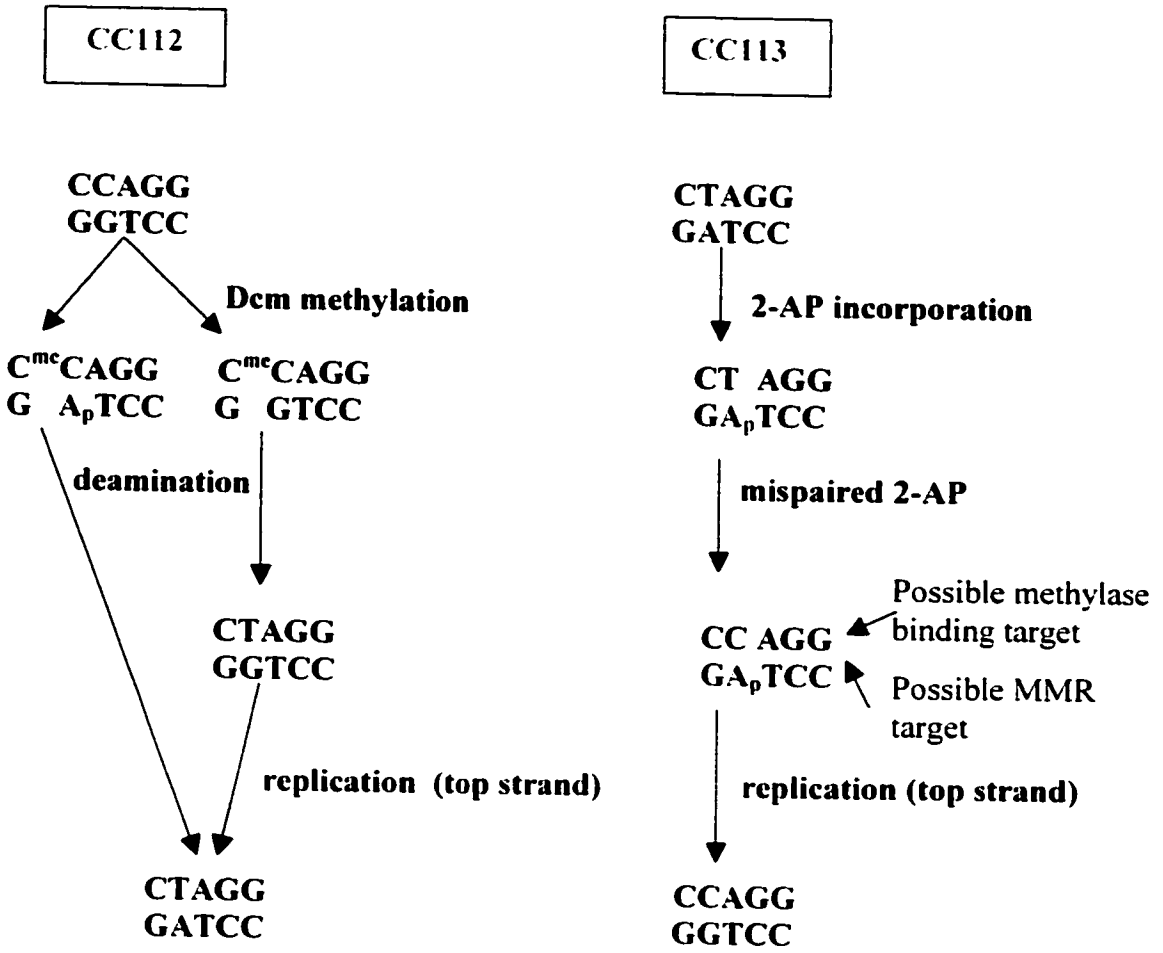


Figure 10. CC112 and CC113 monitoring systems.

^{me}C= methylated cytosine, A_p= 2-aminopurine

3.2.1. 2-aminopurine induced transition mutations are due in part to Dcm

Lac reversion assays were performed with wildytpe cells, CC113, and with an isogenic strain deficient in the cytosine methylase, CC113Δ. These cells were treated with increasing concentrations of 2-aminopurine. Results shown in Figure 11 clearly indicate that the *dcm* deficient strain, CC113Δ, leads to an increase in 2-aminopurine induced T-to-C transition mutations at a higher concentration of the drug than the CC113 strain. There is, therefore, an evident shift between the two strains. Similar methylase effects leading to a shift in dose dependent mutagen curves were also observed with 5-azacytidine (Doiron *et al.*, 1999). Also, these results support Miller's original observation that CCAGG sites are hotspots for 2-aminopurine induced mutations (Coulondre *et al.*, 1978b).

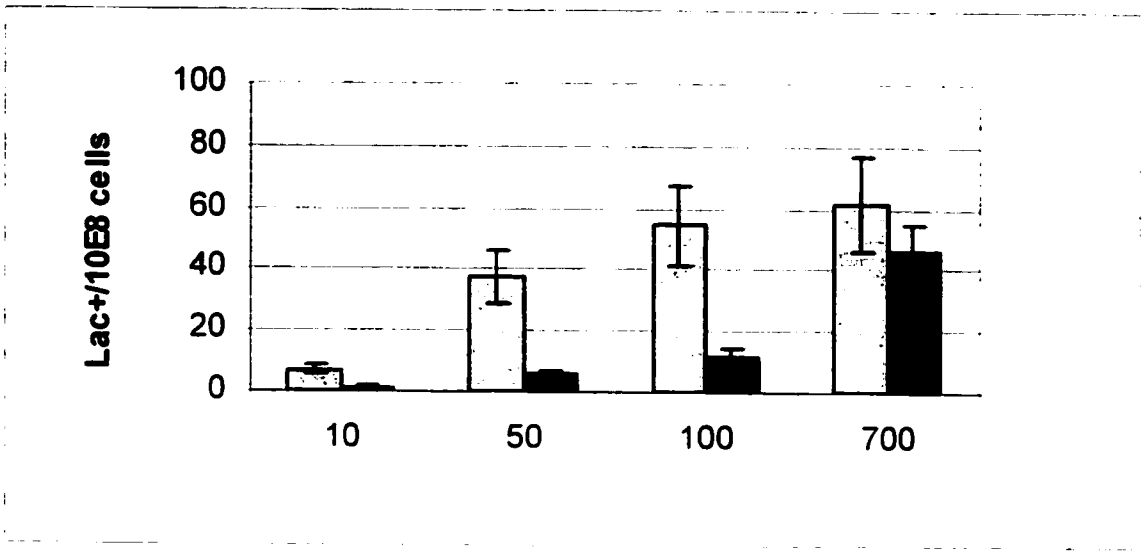


Figure 11. Effect of treating CC113 and CC113Δ with increasing doses of 2-animopurine. Lactose revertants/10^8 cells are monitored over a wide range of 2-aminopurine concentrations (10, 50, 100, and 700μg/ml) . The CC113 strains are represented by the grey bars and the CC113Δ by the black bars.

3.2.2. 2-aminopurine induced C/A mismatches are not reduced by MMR proteins

To determine whether the MutH, MutL, and MutS proteins could access the 2-AP-C lesion in a strain expressing the cytosine methylase, an extra amount of mismatch repair proteins were added to CC113 cells by transforming with plasmids containing the *mut* genes. CC113 monitors CTAGG-to-CCAGG mutations at position 461 in the *lacZ* gene, therefore, changing the phenotype from Lac^- to Lac^+. CC113 cells were transformed with pACYC184 (the empty vector), pMQ348 (H), pMQ339 (L), and pMQ341 (S). These transformed cells were treated with 10μg/ml, 50μg/ml, or 100μg/ml of 2-aminopurine or were left untreated. Lac revertants per 10^8 cells were calculated and are shown in Figure 12. The results indicate that C/A lesions caused by the drug and occurring within this *dcm* recognition sequence are not reduced by increased amounts of mismatch repair proteins suggesting that Dcm may be blocking the site.

To help pinpoint the role of the methylase in these experiments, similar Lac reversion experiments were performed for CC113Δ strains. Here, the purpose of the assay is to confirm if the absence of the methylase, Dcm, allows the C/A mismatch in this sequence context to be accessed by the MMR system. Figure 13 shows the Lac reversion data which confirms that the MMR proteins MutH and MutS are not acting on the site of the mispair. The results for MutL indicate that it may be acting on the mispair, since the addition of the pMutL plasmid has a small effect in reducing the mutations caused by these C/A mismatches. However, typical complementation assays of this kind have previously depicted a reduction in mutations with pMutH as well as pMutL (Macintyre *et al.*, 1997), therefore, the mechanism for the 2-aminopurine induced C→T transition mutations may not be a simple saturation of the MMR system. Finally, put

together, the results for Figure 12 and 13 suggest that 2-AP-C lesions are not subject to MMR.

Similarily, rifampicin resistance assays show that both strains, CC113 and CC113Δ, with the added MMR proteins did not reduce the mutations induced by 2-aminopurine (data not shown). 2-aminopurine has previously been reported to saturate MMR and, therefore, result in an increase in transitions and frameshift mutations (Cupples *et al.*, 1990; Cupples and Miller, 1989). Our lab has reported the reduction of 2-aminopurine-induced frameshifts by the addition of MMR proteins MutH and MutL (Macintyre *et al.*, 1997). However, although 2-AP causes both transition and frameshift mutations, the rifampicin resistance assay only detects the transitions, because the frameshift mutations would yield unviable cells. Thus, the results of these experiments also indicate that 2-AP induced transitions are not a result of saturation of MMR.

Furthermore, the mutations at CCAGG sites in the *rpoB* gene do not lead to rifampicin resistant cells (Jin and Gross, 1988), therefore the rif resistant colonies are not subject to MMR at non-methylatable sites further supporting the hypothesis that Dcm does not block repair of 2-AP-C lesions.

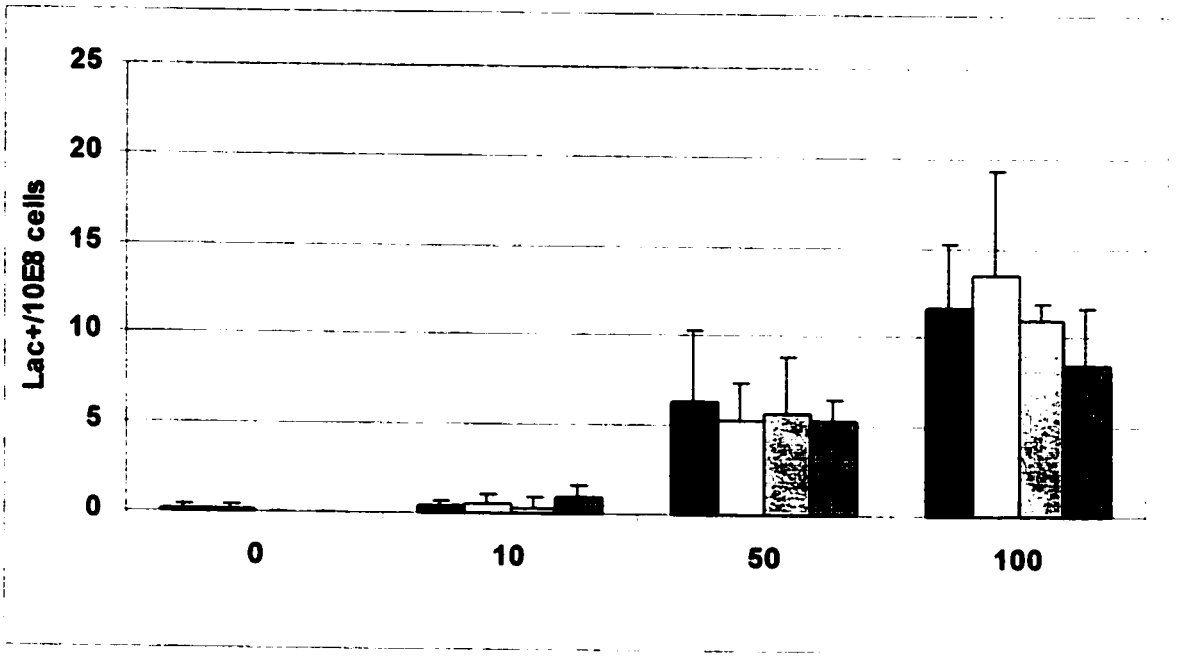


Figure 12: Effect on 2-aminopurine induced T-to-Cs in cells with increase amounts of MMR proteins. Lac reversion assay monitoring T→C mutations in CC113 cells containing plasmids pACYC184 (black bar), pMutH (white bar), pMutL (grey bar), or pMutS (striped bar) treated with various concentrations of 2-aminopurine.The concentrations are, from left to right. 0μg/ml. 10 μg/ml. 50 μg/ml, and 100 μg/ml)

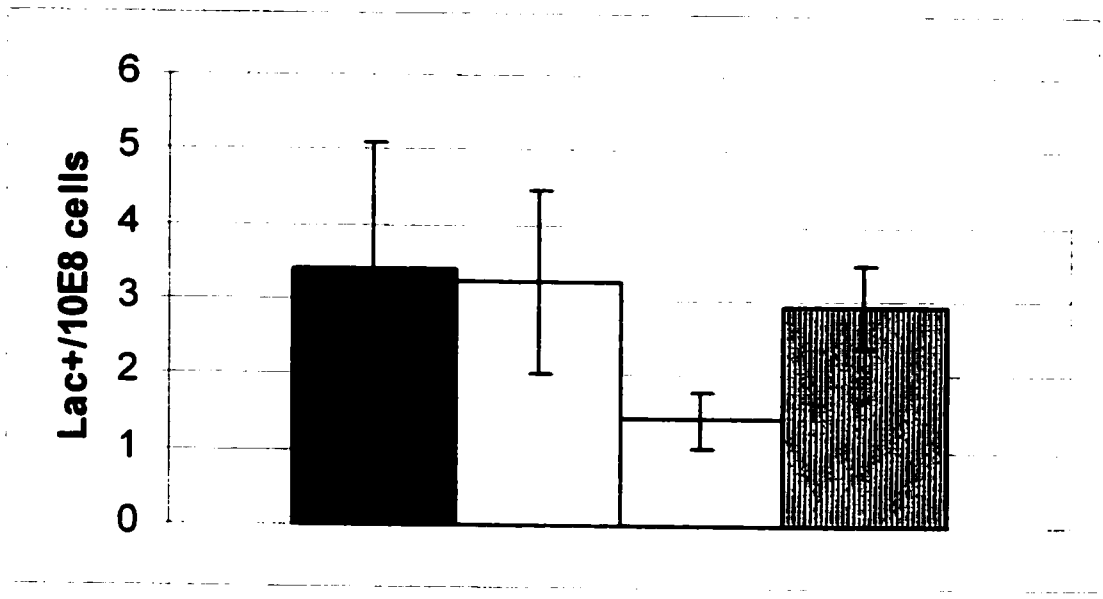


Figure 13: Effect on 2-aminopurine induced T-to-Cs in *dcm* mutants with increasing amounts of MMR proteins. Lac reversion assay monitoring T→C mutations in CC113Δ cells containing pACYC (black bar), pMQ348(H) (white bar), pMQ339(L) (grey bar), and pMQ341(S) (striped bar) plasmids treated with 10μg/ml of 2-aminopurine.

3.2.3. Dcm contributes to C-to-T deamination induced mutations

We have ruled out the possiblity that Dcm blocks access of MMR to 2-AP-C lesions. Now, to test the effects of the Dcm methylation on 2-aminopurine induced C-to-T mutations, CC112 cells were used. The CC112 cells were transformed with plasmids pACYC184, pDCM28, and pDV101, pDV102, pDV105, and pDV106 described in Table 3. Cells were treated with increasing concentrations of 2-aminopurine ranging from 10 μg/ml to 500 μg/ml in duplicate cultures. 10 μl were spotted onto papillation plates as shown in Figure 14A, and in Figure 14B the number of Lac revertants per 10^8 cells are shown for the untreated and 100 μg/ml concentration samples. The results indicate that C-to-T mutations caused by 2-aminopurine occur more readily in cells containing Dcm than those without. This is apparent when comparing the increase in Lac revertants between the cells treated with 2-aminopurine with (Fig 14A and B labeled D^+V^-) and without the methylase (Figure 14A and B labeled D^-V^-). Also the 2-aminopurine increase in cells devoid of the methylase lead to a smaller increase of Lac revertants than those in cells with Dcm present.

Vsr is efficient in repairing *dcm*-induced C-to-T mutations as can be seen by comparing lanes labeled D^+V^- with D^+V^+ in Figure 14A and B. There is a distinct decrease in mutation levels in the strain expressing *vsr*.

The pDV105 and pDV106 plasmids express mutant methylases which bind but do not methylate the cytosine. The results in Figure 14A and B lanes labeled mD^+V^- and mD^+V^+ indicate that the binding is not sufficient for the Dcm-induced mutations seen in D^+V^- since these mutant methylases display mutation rates comparable to the plasmid only control D^-V^- cells. These results further strengthen the hypothesis that Dcm does not

block MMR at these methylatable sites. Rather, that cells containing methylcytosines lead to many more 2-AP-induced C-to-T mutations than those without Dcm, indicating that methylcytosines may base pair more frequently with 2-AP than would normal cytosines.

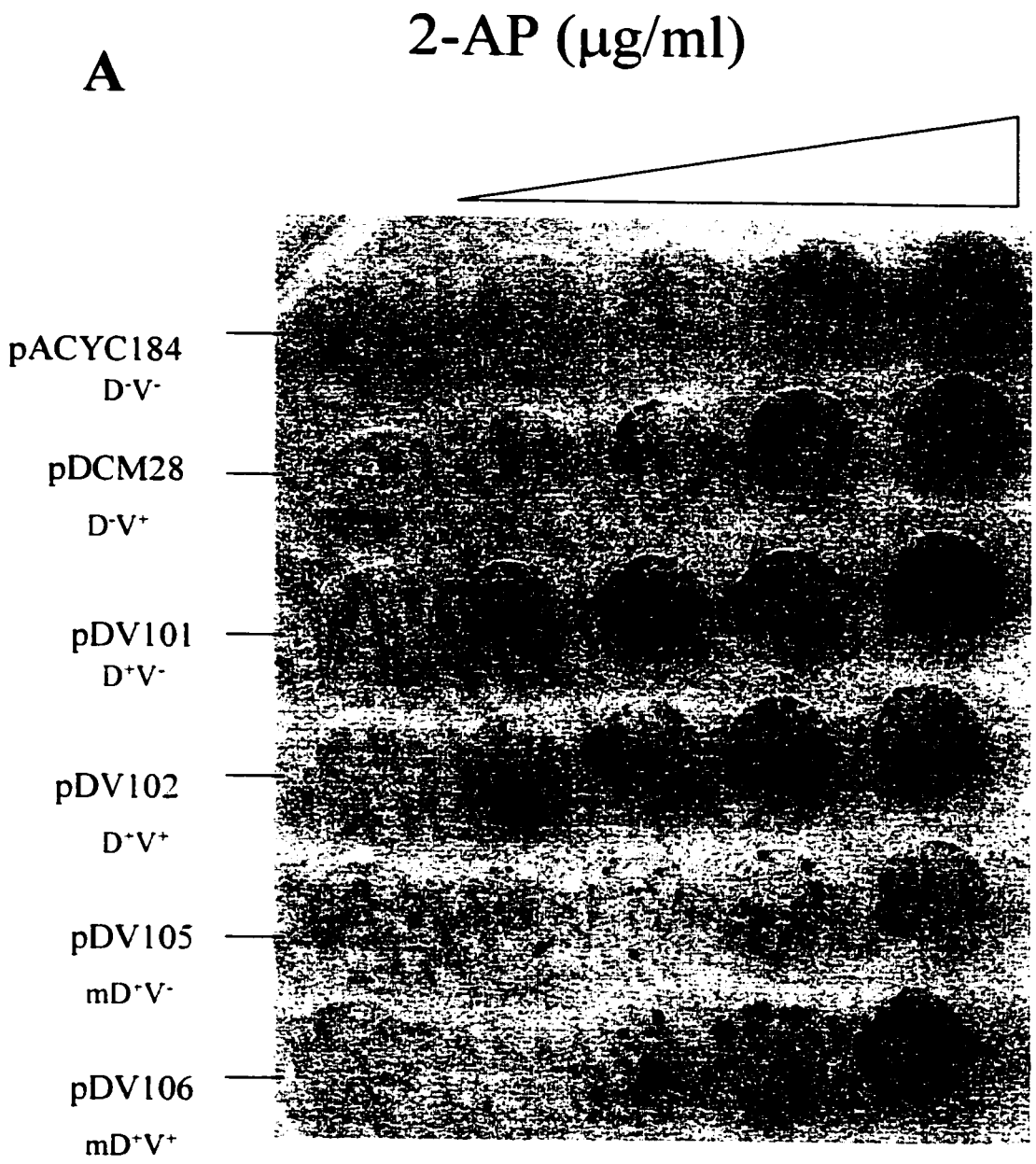


Figure 14A: 2-aminopurine induced C-to-T mutations in cells with various gene expressing plasmids. Papillation assay of C-to-T Lac revertants in CC112Δ strain containing, from top to bottom, pACYC184, pDCM28, pDV101, pDV102, pDV105, and pDV106. Strains were treated (from left to right) with 0, 10, 50, 100 and 500 μg/ml of 2-aminopurine.

B

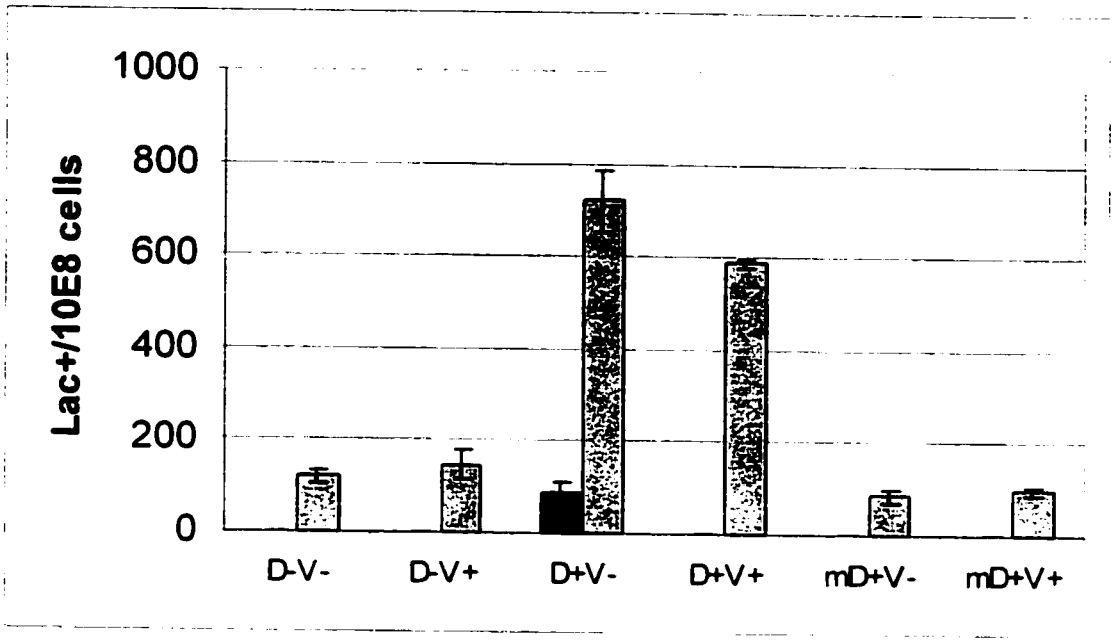


Figure 14B: Lac reversion assay monitoring C→T mutations in CC112Δ cells containing (from left to right) pACYC184, pDCM28, pDV101, pDV102, pDV105, and pDV106 plasmids untreated (black bars) and treated with 100μg/ml (grey bars) of 2-aminopurine.

3.2.4. Effect of 2-aminopurine induced C-to-Ts in MMR deficient mutants

We have tested Dcm's effect on methylatable sites in the presence of 2-aminopurine. We have also tested Dcm's effect and MMR's effect on non-methylatable sites. MMR's effect on 2-aminopurine-induced C-to-T mutations at methylatable sites remains. To test this, CC112 cells were used to monitor the C-to-T mutations induced by 2-aminopurine. CC112*mutH*, CC112*mutL*, and CC112*mutS* cells were constructed and treated with the drug and compared to the wildtype strain to test whether the cells devoid of the MMR system affect the frequency of mutations caused by 2-aminopurine. Figure 15A, B and C show the C-to-T mutations as described previously. The elimination of the *mut* genes does not affect the frequency of mutations caused by 2-aminopurine as can be observed by comparing the CC112*mut* strains Figure 15A lanes 2-4 and Figure 15C. Since no further dose dependent increase or shift is noted over that of the wildtype cells, these results continue to support the hypothesis for 2-AP-C lesions not being subject to MMR.

The small increase noted with the untreated samples of the CC112*mut* strains compared to the wildtype CC112 strain (Figure 15B) indicates that there is an effect on removing the repair system and that the system does in fact act on that site. However, the increase is not further affected by 2-aminopurine.

A

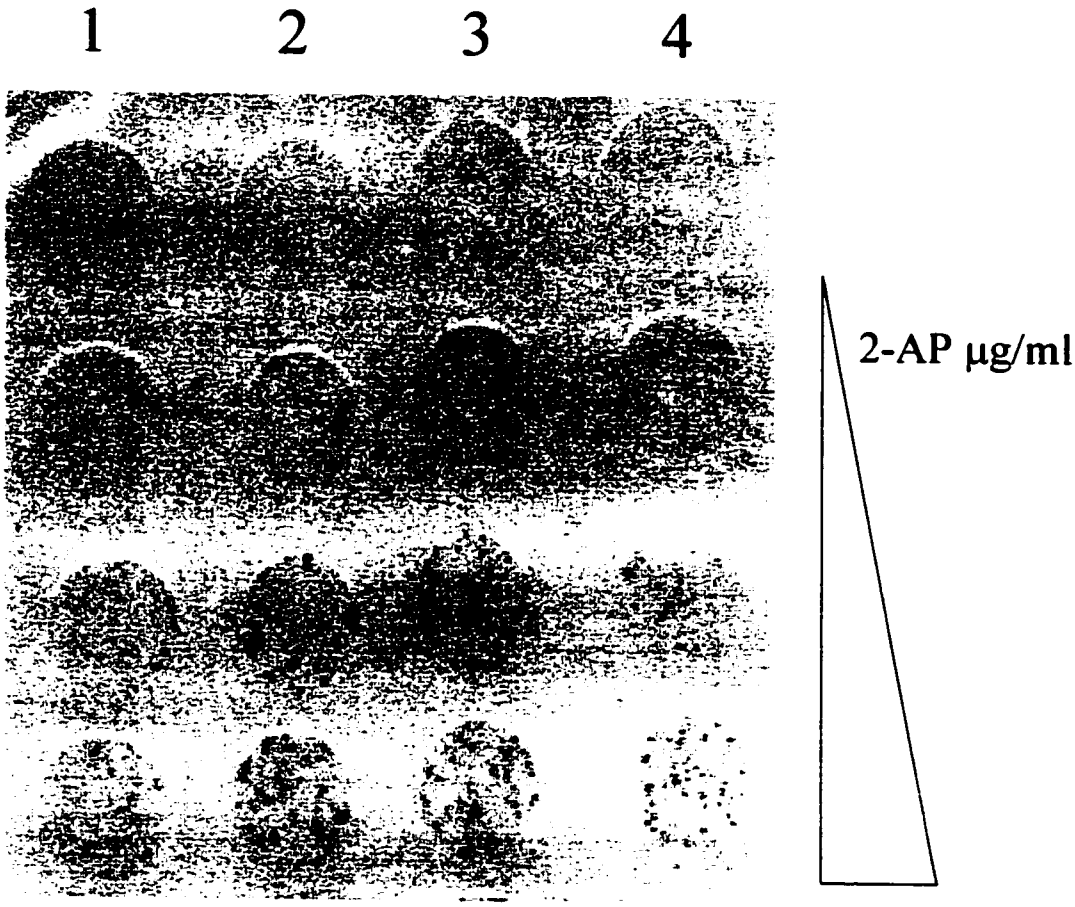
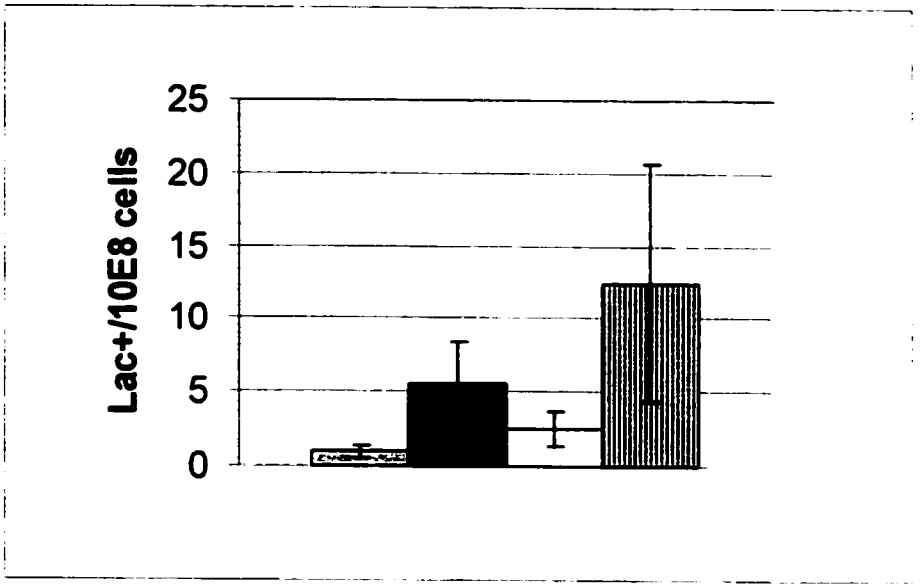


Figure 15A: 2-aminopurine induced C-to-Ts in mismatch repair deficient strains. Papillation assay of strains CC112 (lane 1), CC112*mutH* (lane 2), CC112*mutL* (lane 3), and CC112*mutS* (lane 4). Strains were treated with 0, 10, 50 and 100 µg/ml of 2-aminopurine.

B



C

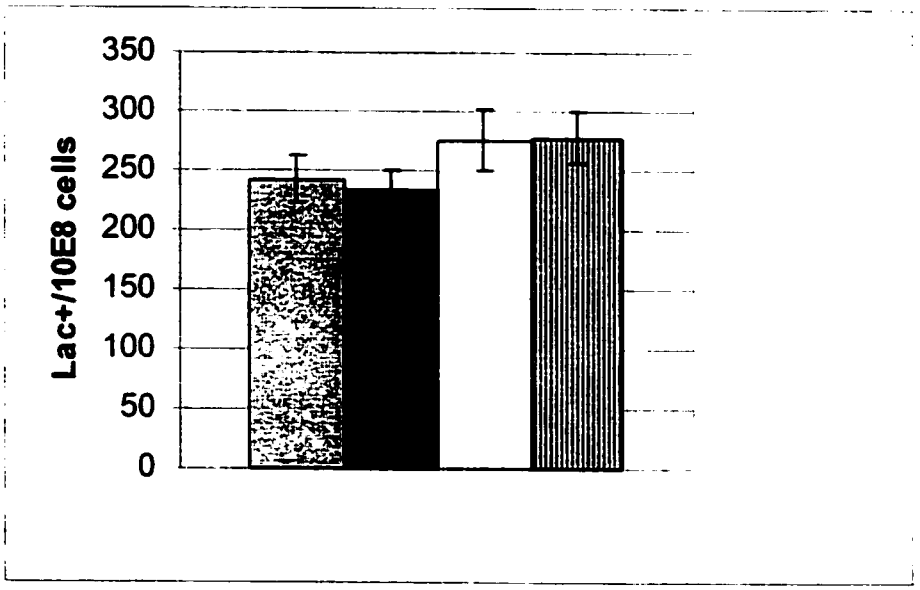


Figure 15: Lac reversion assay to detect C→T mutations induced in mismatch repair deficient strains and wildtype cells. Panel **B** are (from left to right) CC112, CC112*mutH*, CC112*mutL*, and CC112*mutS* cells untreated. Panel **C** are (from left to right) CC112, CC112*mutH*, CC112*mutL*, and CC112*mutS* cells treated with 100μg/ml of 2-aminopurine.

3.3. Effect of 5-azacytidine on mutagenesis and repair

Recently, it has been shown that C-C mispairs in *Schizosaccharomyces pombe* were corrected by nucleotide excision repair (Fleck *et al.*, 1999;Sancar, 1999). This mispair is a poor substrate for the mismatch repair system of *E.coli*, so the possibility that there is an alternative repair system in *E.coli* to deal with these mispairs is feasible. The C-to-G mutations caused by 5-azacytidine can only occur due to a C-C or G-G mispair and since 5-azacytidine is a cytidine analogue which is believed to be incorporated into the DNA as 5-azacytosine (Friedman, 1985), 5-azaC-C mispair is the probable candidate.

3.3.1. 5-azacytidine lesions which cause C-to-G mutations are repaired by NER

To determine whether NER is involved in the prevention of C-to-G mutations, NER mutants, CC103*uvrA*, CC103*uvrB*, and CC103*uvrC* were constructed and verified by sensitivity to ultraviolet light (Figure16). A Lac reversion assay was performed to detect the 5-azacytidine induced C-to-G mutations for these NER deficient mutants (Figure 17). The results indicate that the UvrA, UvrB and the UvrC proteins of the NER system are involved in the repair of the lesions, since in their absence the C-to-G mutations increase 1.1-3 fold in the same dose-dependent manner. The increases vary in their extent over that of the wildtype strain; however, the increase is consistent and reproducible. Specifically, the *uvrB* strain was the most variable, however, the increase in mutations at low concentrations of 5-azacytidine with this mutant is always consistent even though the extent of the increase fluctuated between experiments. Also, this mutant is the most variable at the peak concentration of 50 μg/ml with some experiments being well above the wildtype and others, like in Figure 17, showing no difference.

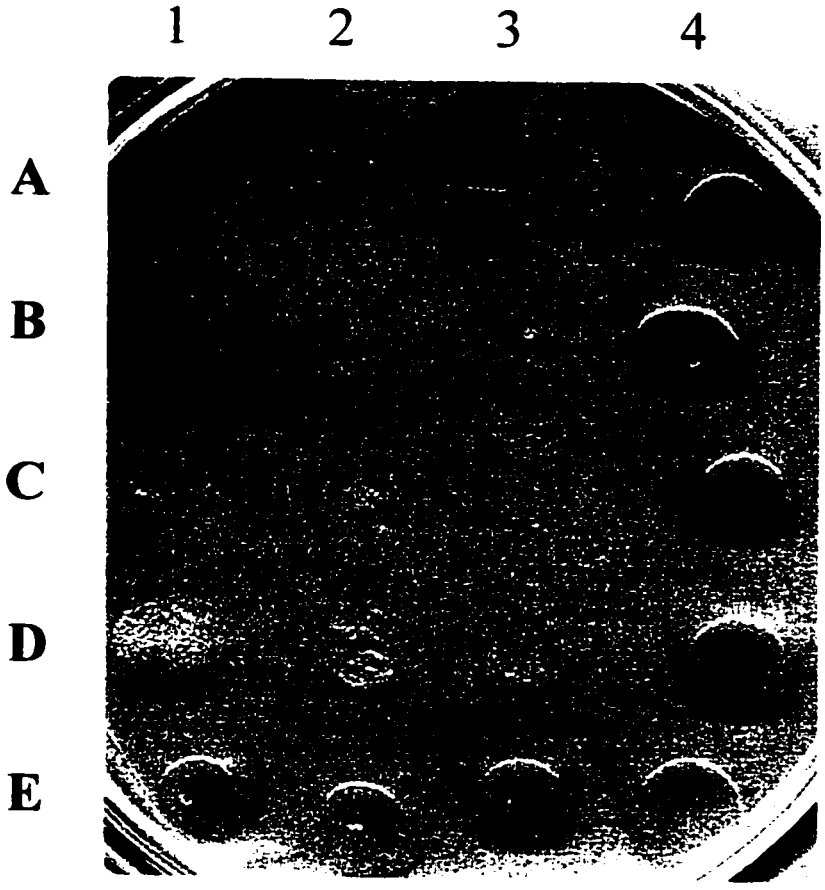


Figure 16: Testing NER deficient strains for ultraviolet sensitivity. Lane 4 contains CC103 wildtype cells. Lanes 1, 2, and 3 contain CC103*uvrA*, CC103*uvrB*, and CC103*uvrC* cells respectively. A, B, C, D, and E represent 30, 10, 5, 1, 0 seconds of exposure to ultraviolet light respectively.

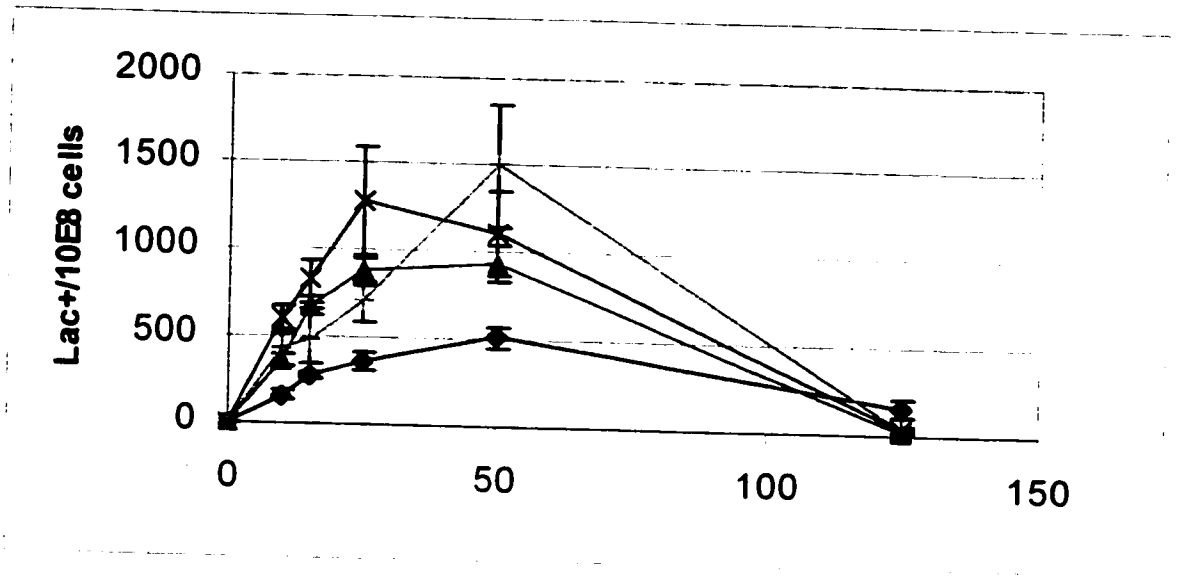


Figure 17. 5-azacytidine induced C-to-G mutations in nucleotide excision repair deficient strains. Lac^+ revertants/10^8 cells are monitored with increasing concentrations of 5-azacytidine. The values shown are +/- SEM.The widtype cells CC103 are represented by a (◆), the CC103*uvrA*$^-$ by (▲), the CC103*uvrB*$^-$ strain by (✕), and the CC103*uvrC*$^-$ cells are the (+).

The response of *uvr* mutants to 5AC was also observed by another group (Bhagwat and Roberts. 1987), however, no mutational data were presented. The results for that study included toxicity data which implicated *uvrA* in the correction of 5-azacytidine-induced damage since it was more sensitive to the drug than wildtype cells. Therefore, our study not only supports these data but also provides the possibility of the identification of the lesion as well as a more complete analysis of which components of the NER repair system are involved.

3.3.2. MutS and MutL are needed for the repair of 5-AC induced mutations

The experiments for detecting the C-to-G mutations were repeated with mismatch repair deficient strains. The constructed *mut* strains were tested for their mutator phenotype (Figure 18). The Lac reversion assay results (Figure 19) show increases in the specific mutation associated with 5-azacytidine for the *mutL* and *mutS* mutant strains. These increases are dose dependent and different from one another. The mutS strain causes a 2 fold increase at lower 5-azacytidine concentrations whereas the *mutL* strain peaks at a higher concentration at a point where the *mutS* is actually not above that of the wildtype. The *mutH* mutant does not result in an increase of these dose dependent mutations but rather shows an unexpected 2-fold decrease throughout all the concentrations. Therefore, the mismatch repair system is not solely responsible for repairing the lesions caused by the methylase independent effect of the drug. In other words, the difference between the *mutL,S* and *mutH* strains indicates that the three components are not involved in a cooperative system for repairing this type of lesion.

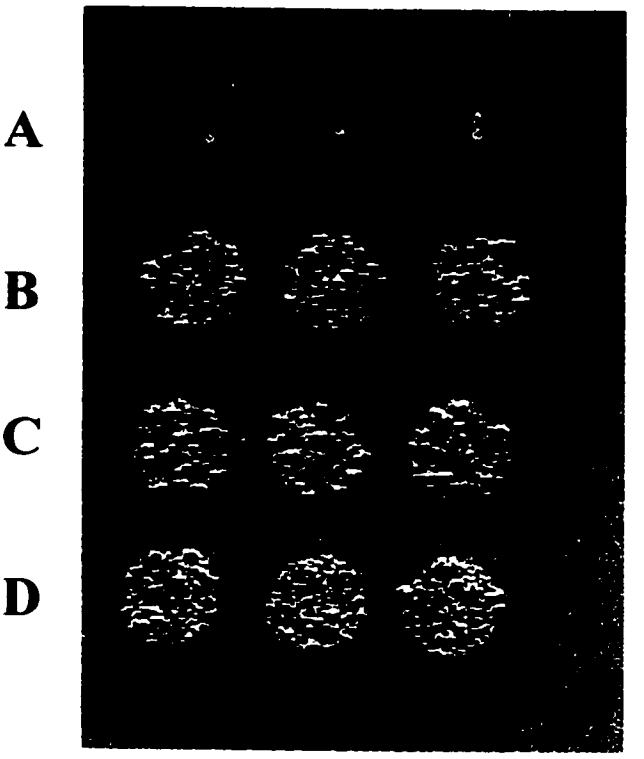


Figure 18: Testing MMR deficient strains for rifampicin resistance. In row **A**, 10µl of saturated CC103 wildytpe cells were spotted in triplicates onto a rifampicin containing LB plate. In rows **B**, **C**, and **D**, 10 µl of saturated CC103*mutH*, CC103*mutL*, and CC103*mutS* cultures were spotted in triplicates.

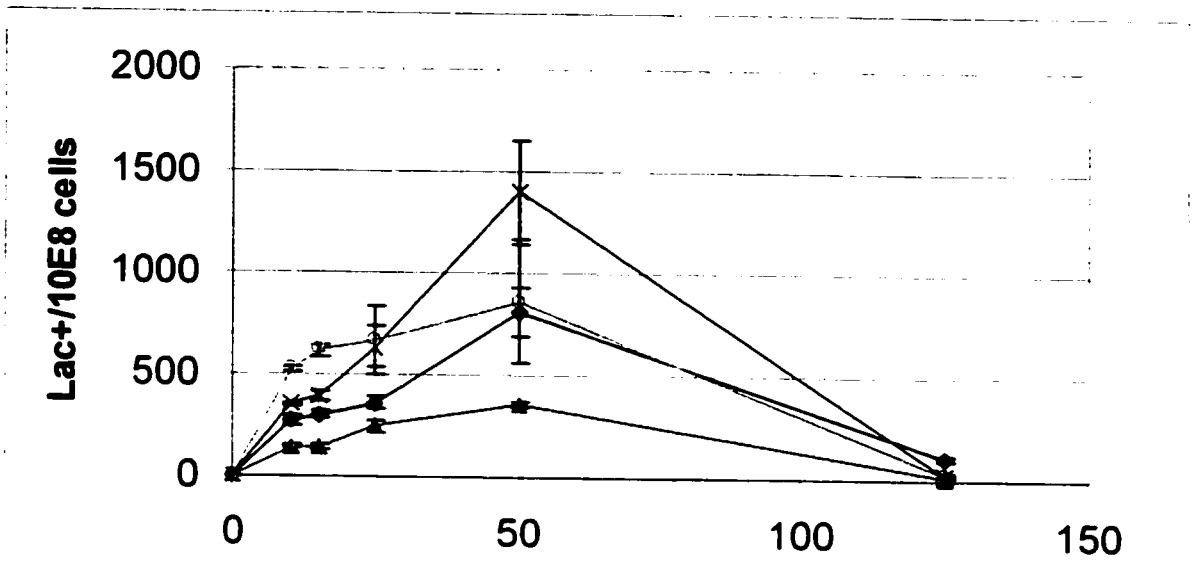


Figure 19. 5-azacytidine induced C-to-G mutations in mismatch repair deficient mutants. Lac^+ revertants/10^8 cells were monitored over a range of 5-azacytidine concentrations (0,10,15,25,50, and 125μg/ml). The values +/- the SEM are shown.The wildtype cells are show by (◆), the CC103mutH cells by (▲), the CC103mutL by (✕), and the CC103mutS by (○).

In fact, the decrease shown by the *mutH* strain indicates that maintaining the MMR system together as a complete group actually interferes with the correction of this 5-azacytidine induced lesion.

Also, the fact that the lack of some of the components of mismatch repair do not lead to an increase in mutation rate supports the notion that G-G may not be the mispair caused by 5-azacytidine since this mismatch is known to be a good substrate for MMR. Also, for this same reason, the mismatch repair deficient strains would display an increase in the background C→G mutations in the absence of the drug. The increasing mutations seen with deficient strains of both post-replicative repair systems indicates that certain components of both repair systems could be responsible for the repair of this drug dependent lesion. To further investigate this hypothesis, plasmids were constructed with the *uvrA*, *uvrB*, and *uvrC* genes cloned in the bacterial two-hybrid system.

3.3.3. 5-AC induced C-to-Gs are not due a nucleotide pool imbalance

To ensure that increases in C-to-G caused by 5-AC were not due to a nucleotide pool imbalance, cells were grown in the presence of increasing concentrations of cytidine, cytosine, or 5-azacytidine. Neither cytidine nor cytosine caused any Lac^+ revertants (Table 5). This result strengthens the evidence for the specific effect of the 5-azacytidine drug on C→G mutations.

concentration	5-azacytidine	cytidine	Cytosine
0.01mM	80.20	<0.52	<0.57
0.04mM	139.75	<0.53	<0.57
0.10mM	360.82	<0.52	<0.59

Table 5. Effect of nucleotide pool imbalance on 5-azacytidine induced C-to-G mutations. Shown are Lac^- revertants/10^8 cells. The strain used for the reversion assay is CC103.

3.3.4. Bacterial two-hybrid interactions

The bacterial two-hybrid system (Karimova *et al.*, 1998) was used to look at the interactions between the mismatch repair proteins and the nucleotide excision repair proteins. In this assay, interactions between two proteins of interest bring together two domains of the catalytic subunit of the *Bordetella pertussis* adenylate cyclase (T18 and T25), resulting in synthesis of cAMP. In a *cyaA* background, this allows activation of the CAP binding protein, stimulating transcription of a number of sugar operons including *lac* which was used as the reporter gene for our study. The MutH, MutL, MutS, UvrA, UvrB, and UvrC proteins were expressed as fusion proteins with the adenylate cyclase catalytic domain using this two-hybrid system. The 5' end of the gene of interest is cloned downstream of the pT25's adenylate cyclase catalytic domain gene sequence. Similarily, in the pT18 vector the 3' end of the gene of interest is cloned in frame and upstream to the adenylate cyclase catalytic domain sequence. All the possible pair wise combinations were tested. The *uvr* clones were tested for complementation by transforming them into their *uvr*$^-$ strain counterparts (Figure 20). The results indicated that the pT25 series, pACYC based plasmids, complemented the UV sensitivity phenotype rendering them UV resistant, but that the clones that were in the pT18, pBR based plasmids, did not complement, and these transformed strains remained UV sensitive. These results may suggest that the N-terminal domain of the Uvr proteins interacts with the other proteins involved with the nucleotide excision repair proteins, whereas the C-terminus of UvrB interacts with the Mut proteins. Another possibility is that the fusion protein's conformation is not sufficient for function but is enough for interacting with other proteins.

Studies (Mansour *et al.*, 2001) showed interactions between mismatch repair proteins using the bacterial two-hybrid system and also reported that the pT18 fusion plasmids are not active in their repair function, while the pT25 fusion plasmids did complement the mismatch repair deficient phenotype. These similar results suggest an internal problem with the pT18 plasmids. However, the pT18-MutH, MutL, and pMutS plasmids still showed interactions in both studies supporting the choice in using them in these studies.

The interactions that were detected were UvrB-MutL and UvrB-MutS. These are the pT18-Uvr proteins with the pT25 series Mut proteins (Figure 21 labeled 4 and 5). The interactions shown in this figure are 5-azacytidine dependent. The concentration of 5-azacytidine used was 10μg/ml, which is enough to cause C→G mutations but not so high as to cause toxicity. Also to show that the interaction noted was correlated with the drug induced lesions, cells containing the pT18 UvrB and pT25 MutL plasmids were treated with increasing concentrations of the drug. The result is shown in Figure 22. Also, another novel interaction seen in these two-hybrid experiments was pT18-UvrC with pT25-MutL. This interaction was observed in the absence of the drug (Figure 21 labeled 9).

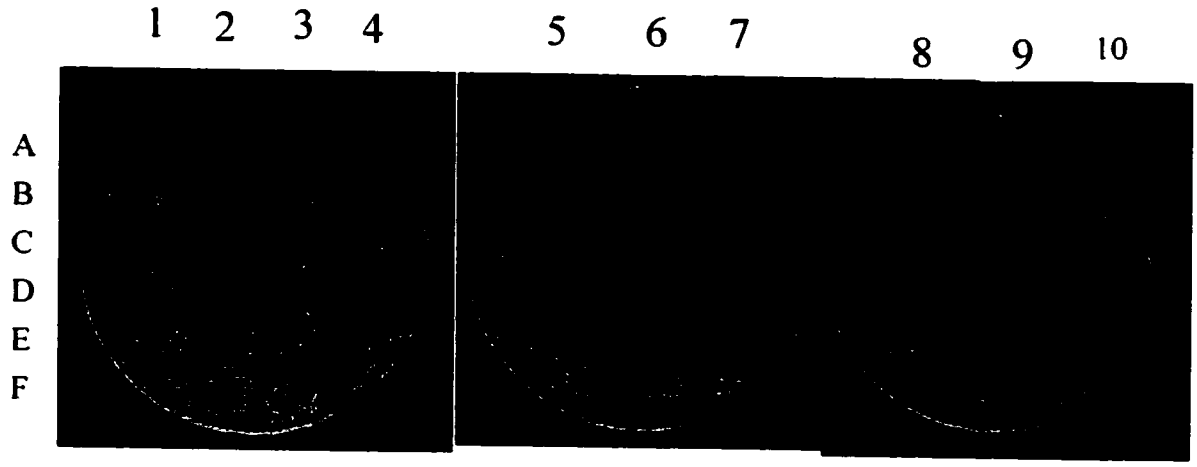


Figure 20. Complementation of bacterial two-hybrid vectors. Testing the Uvr-T18 and Uvr-T25 fusion proteins for repair proficiency. Plasmids containing *uvrA*, *uvrB*, or *uvrC* genes were tested for their ability to complement the ultraviolet light resistance phenotype. Sample 2, 5, and 10 are the *uvrA*-T25, *uvrB*-T25 and *uvrC*-T25 plasmids transformed into *uvrA*, *uvrB*, and *uvrC* respectively. Samples 3, 6, and 9 are T18-*uvrA*, T18-*uvrB*, and T18-*uvrC* respectively. Samples 4, 7, and 8 are strains *uvrA*, *uvrB*, and *uvrC* respectively. A,B,C,D,E, and F represent 60, 30, 15, 10, 1, 0 seconds of exposure to ultraviolet light, respectively.

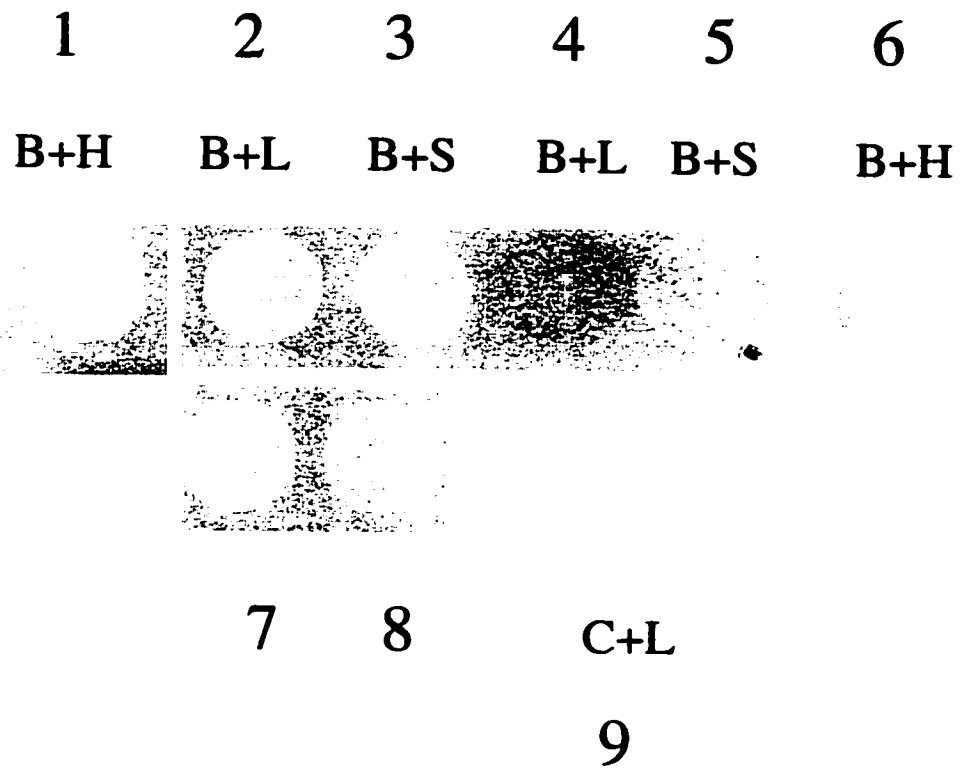


Figure 21. Bacterial two-hybrid interactions between nucleotide excision repair and mismatch repair proteins. The first letter labeled above refers to the uvr gene in the pT18 plasmid and the second to the mut gene in the pT25 plasmid. Samples for lanes 4-6 were grown in the presence of 10 μg/ml of 5-azacytidine. Sample 7 is the empty plasmids pT18 and pT25 and 8 is the empty plasmids in the presence of 10μg/ml of the drug.

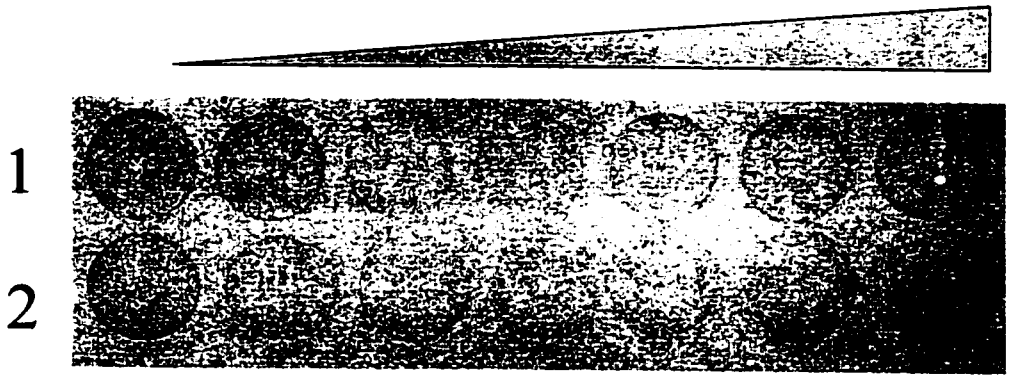


Figure 22. Interaction of UvrB and MutL is dose-dependent.
10 μl spots are shown for LJ2809 cells containing empty bacterial 2-hybrid plasmids (Row 1)pT18+pT25. Row 2 are cells containing bacterial 2-hybrid plasmids uvrB-T18 and mutL-pT25. The concentrations of 5-azacytidine are, from left to right, 0, 1.5, 2.5, 3.5, 5, 10, and 20 μg/ml.

3.3.5. Interactions between Mut and Uvr proteins do not result in saturation of MMR

If various components of MMR and NER interact and form another repair system to recognize and repair the 5-azacytidine induced lesion then it is possible that errors in DNA replication, which are targets for the mismatch repair system, should remain unrepaired due to a saturation of the mismatch repair proteins. The saturation should lead to an increase in both transition and frameshift mutations (Macintyre *et al.*, 1997). An increase in Lac^+ revertants was not seen using CC102 as an indicator for the G→A transition mutation (data not shown) consistent with previous results (Cupples and Miller, 1989). CC107 cells treated with increasing doses of 5-azacytidine did lead to a small but significant increase in frameshift mutations (Figure 23). This increase is dose dependent; however, it is not as high as previously reported for saturation of the MMR repair systems (Macintyre *et al.*, 1997). Also, it is not as high a mutation rate as one that would occur in the absence of any one of the main genes of this mismatch repair system.

3.3.6. dinB is not responsible for 5-azacytidine induced frameshift mutations

It has been suggested that the product of the *dinB* gene, DNA polymerase IV, contributes to an increase in spontaneous mutations (Strauss *et al.*, 2000). The specific types of mutations noted are frameshifts and base substitutions. *dinB* is induced as part of the SOS response. It was further proven that cells devoid of *dinB* actually show a decrease in frameshift mutations (Strauss *et al.*, 2000). It is, thus, of interest to determine

if the frameshift mutations observed in cells treated with 5-azacytidine, which has been previously shown to be a weak inducer of the SOS response, are caused by the auxiliary action of the DNA polymerase IV. To do this, a dinB mutant strain and a wildtype control strain, were treated with increasing doses of 5-azacytidine. The results shown in Figure 23 do not support the possibility that the *dinB* product is responsible for the drug dependent frameshift mutations.

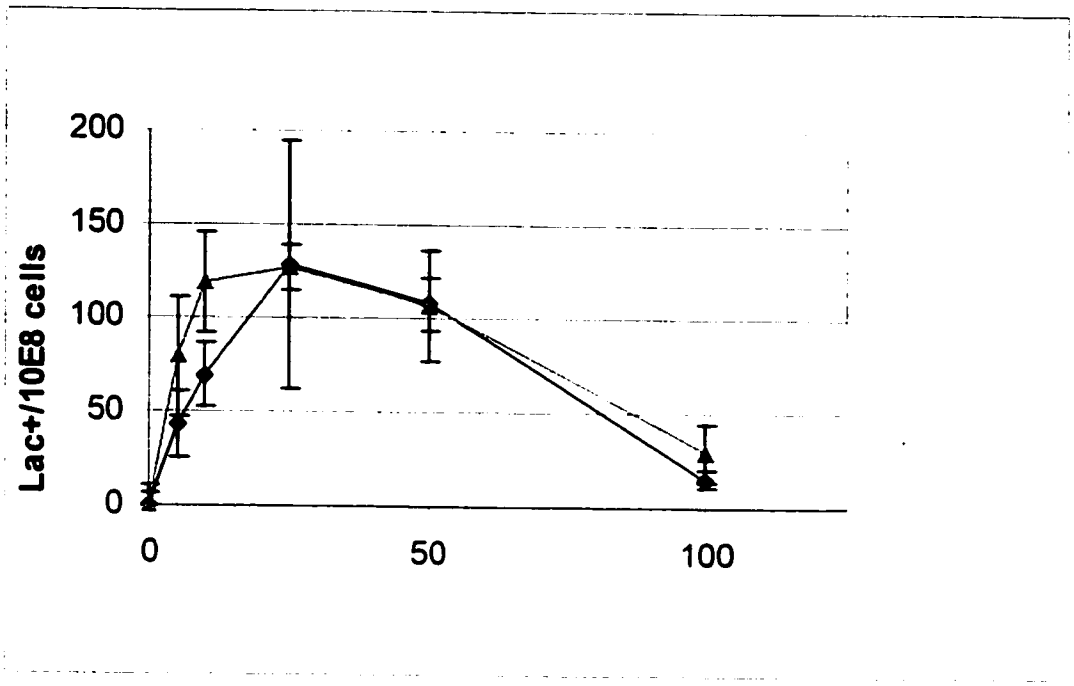


Figure 23. 5-azacytidine induced frameshift mutations in cells deficient in polymerase IV. Lactose revertants/10^8 cells were calculated for both wildtype cells CC107 (◆) and CC107*dinB* (▲) cells. Concentrations of 5-azacytidine used are 0, 10, 15, 25, 50, and 100 μg/ml.

3.3.7. The frameshifts induced by 5-AC are not a result of the Dcm lesion or Vsr

Dcm is *E.coli*'s only cytosine methyltransferase. It methylates the second cytosine in the sequence CCA/TGG. However, when 5-azacytidine is the target of the methylation the methylase remains trapped in a covalent complex with the DNA (Santi *et al.*, 1984). This 5-azacytidine-induced lesion has been shown not to further increase the C-to-G mutations since deficient strains for the *dcm* gene treated with increasing doses of the drug do not lead to an increase in mutations (Doiron *et al.*, 1999). To study whether the 5-azacytidine induced frameshift mutations are linked with the Dcm dependent lesion, cells were constructed to be deficient in the *dcm* gene and to harbor the CC107 episome which monitors the addition of a guanine in a series of six guanines in the *lacZ* gene. The results show that Dcm or the Dcm dependent lesion are not responsible for the increase in the frameshift mutations seen with increasing doses of 5-azacytidine (Figure 24A).

As previously mentioned, high levels of Vsr lead to a saturation of the MMR system which causes increases in transition and frameshift mutations. If Vsr levels are slightly elevated in cells treated with 5-azacytidine perhaps the small increase would be the reason for the small increase in 5-azacytidine dependent frameshifts. Figure 24B shows CC107 cells and the *vsr* deficient isogenic strain treated with increased concentrations of 5-azacytdine. The results show that Vsr is not responsible for the increase in frameshifts caused by the drug.

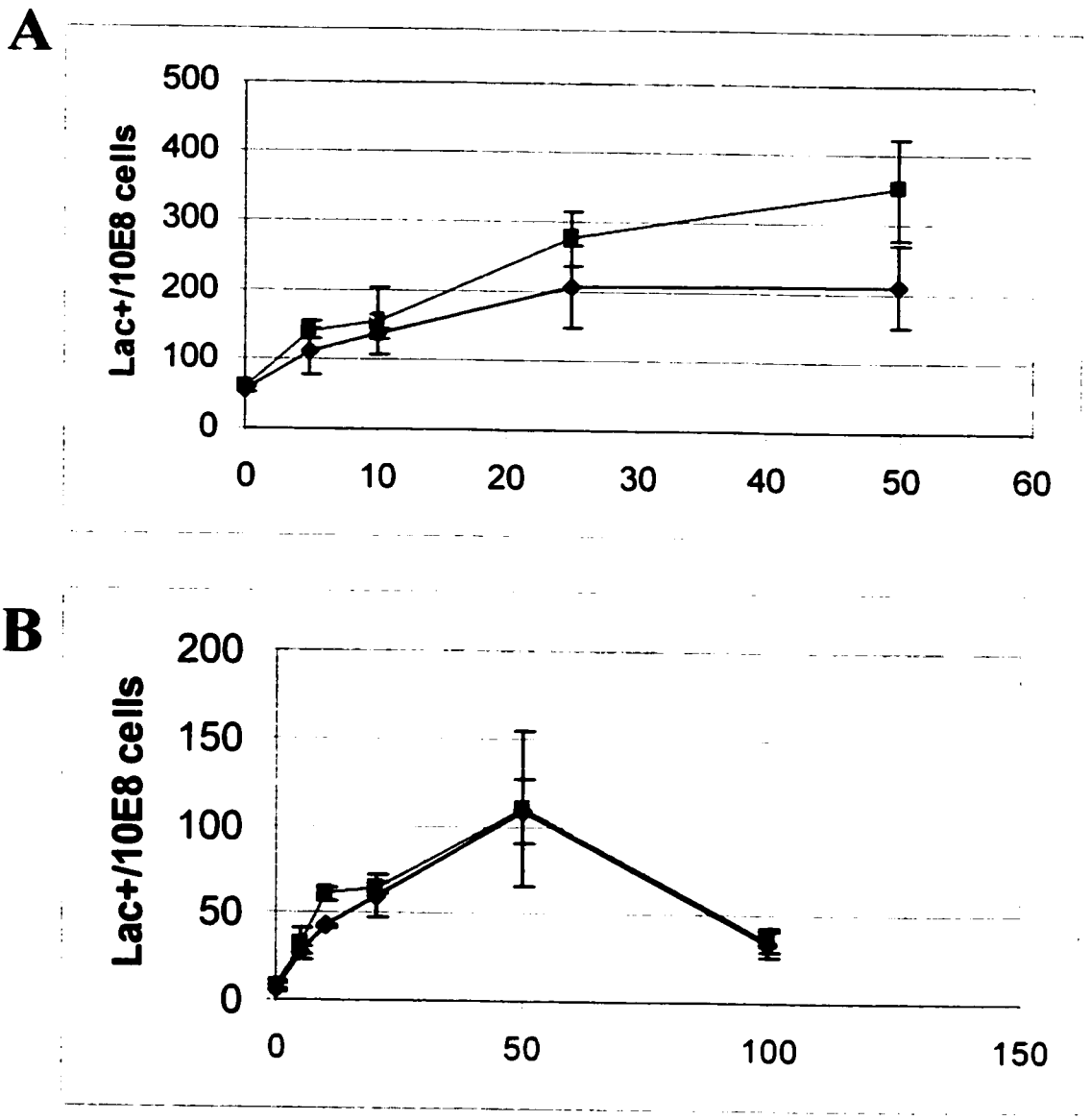


Figure 24. Frameshift mutations caused by 5-azacytidine in *E.coli* cells deficient in *dcm* and *vsr*. Panel **A** shows dose dependent increases in frameshifts with both CC107 (◆) and CC107*dcm* (■) cells when treated with increasing doses of 5-azacytidine (0,5,10,25,and 50μg/ml). Panel **B** shows increases in frameshifts with both wildtype CC107 (◆), and CC107*vsr* (■) cells with 0,5,10,25,50, and 100μg/ml of 5-azacytidine.

3.4. Dcm dependent 5-azacytidine induced lesions

As mentioned above, DNA containing 5-azacytosine has been shown to be bound irreversibly to the DNA cytosine methyltransferase. *Escherichia coli* K12 contains a single cytosine methylase. The methylase is 60 kDa and when covalently bound to DNA containing the analog could interfere with the replication machinery.

3.4.1. 5-azacytidine causes decrease in plasmid replication

To test whether the complex interferes with DNA replication, cells containing a deletion for the *dcm* gene were transformed with either a control plasmid (pACYC184) or a plasmid containing the *dcm* gene under the control of its own promoter (pDV101). This plasmid has been shown to overproduce Dcm (Figure 25 lane D). Strains used for Figure 25 and Figure 26 are different. Both are *dcm* deletion strains, however. Western blot analysis for Figure 25 was performed for another experiment (Doiron *et al.*, 1999). Plasmid DNA was extracted from CC221 cells grown to the same OD_{600}. The levels of the DNA are shown in Figure 26. There is a decrease in plasmid DNA with increasing 5-azacytidine concentrations in the samples where the *dcm* gene is over-expressed from the plasmid (lanes 4-6). However, cells treated with the drug, but lacking the methylase, do not display this decrease in plasmid yield (lanes 1-3).

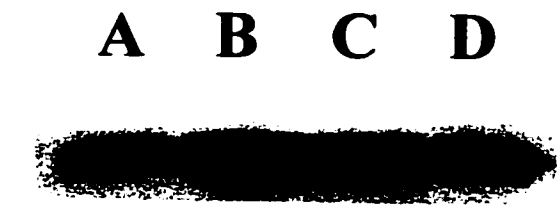


Figure 25: Western analysis of CC112Δ containing plasmids.
Lanes **A**, **B**, **C**, **D**, and **E** contain samples taken from CC112Δ cells expressing pDV102, pDV106, pDV105, pDV101, and pACYC184 respectively. Lane **F** contains sample taken from untransformed CC112Δ cells.

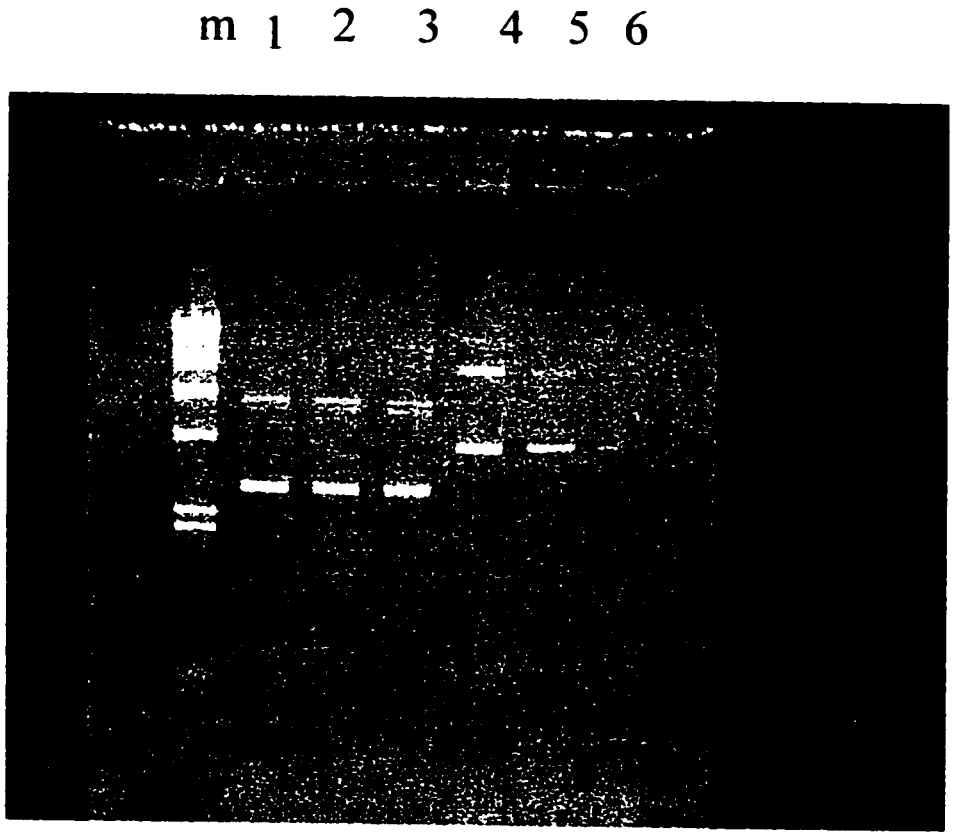


Figure 26. 5-azacytidine induced decrease in plasmid DNA replication. Plasmid DNA preparations were isolated from CC221 cells grown to the same OD_{600}. Equal volumes of the DNA plasmid preparation were electrophoresed onto a 1% agarose gel.Lane labeled "m" contains the λ *Hind*III molecular weight marker. Lanes 1, 2and 3 are pACYC184 plasmid DNA from cells treated with 0, 5, and 10 μg/ml of 5-azacytidine respectively. Lanes 4, 5, and 6 are pDV101 plasmid DNA from cells treated with 0, 5, and 10 μg/ml of 5-azacytidine respectively.

3.4.2. Decrease in DNA replication is dependent on binding of the methylase

We hypothesized that the decreased yields of plasmid DNA were due to a block in DNA replication. To test this hypothesis, mutant methylases were used. The first mutant methyltransferase has a mutation in the active site at position 177. The cysteine amino acid has been changed to a serine which still allowed the methylase to bind to its DNA substrate but which would not allow the methyltransferase to transfer the methyl group (Wyszynski *et al.*, 1992). The second mutant methylase has a mutation at the same 177 position but the cysteine in this case has been changed to a tryptophan and, therefore, the binding is completely inhibited (Wyszynski *et al.*, 1992). Cells devoid of *dcm* were transformed with plasmids expressing the various methylases just described. Figure 27 shows the effect on plasmid DNA yield in cells expressing no methylase (lanes 1 and 2), the wildtype methylase (lanes 3 and 4), the mutant C177S (lanes 5 and 6), and the mutant C177W (lanes 7 and 8). In the cells containing no methylase no difference is noted between the plasmid yield of untreated and treated cells. The cells expressing Dcm showed a decrease in plasmid DNA yield when cells were treated with 5-azacytidine. However, the untreated cells containing Dcm had unaffected plasmid DNA levels, suggesting the overexpression of the methylase is not the reason for the effect, but rather it is due to the Dcm-trapped covalent complex with the 5-azacytosine-containing DNA. Finally, the results for the mutant methylases show that the C177S mutant, which binds DNA but does not methylate it, leads to a decrease in plasmid DNA yield in the treated sample. The decrease seen with C177S supports the hypothesis of the covalent complex causing a block in replication, although the decrease is not as dramatic as that seen with the wildtype methylase. C177W, which neither binds nor methylates DNA, shows no

effect on DNA yield whether the cells were treated with 5AC or not. This confirms the importance of the binding in the Dcm dependent lesion on DNA replication effects.

These experiments were all performed with pACYC based plasmids which contain twelve CCAGG methylase recognition sites. When these experiments were done with pBR322 based plasmids. which contain only six CCAGG sites, the DNA replication effects were not as pronounced (data not shown). This also supports the hypothesis since more complexes in the pACYC based plasmids would lead to more blocks and less replication.

Taken together, the data supports our hypothesis that covalent Dcm-5AC complexes block DNA replication.

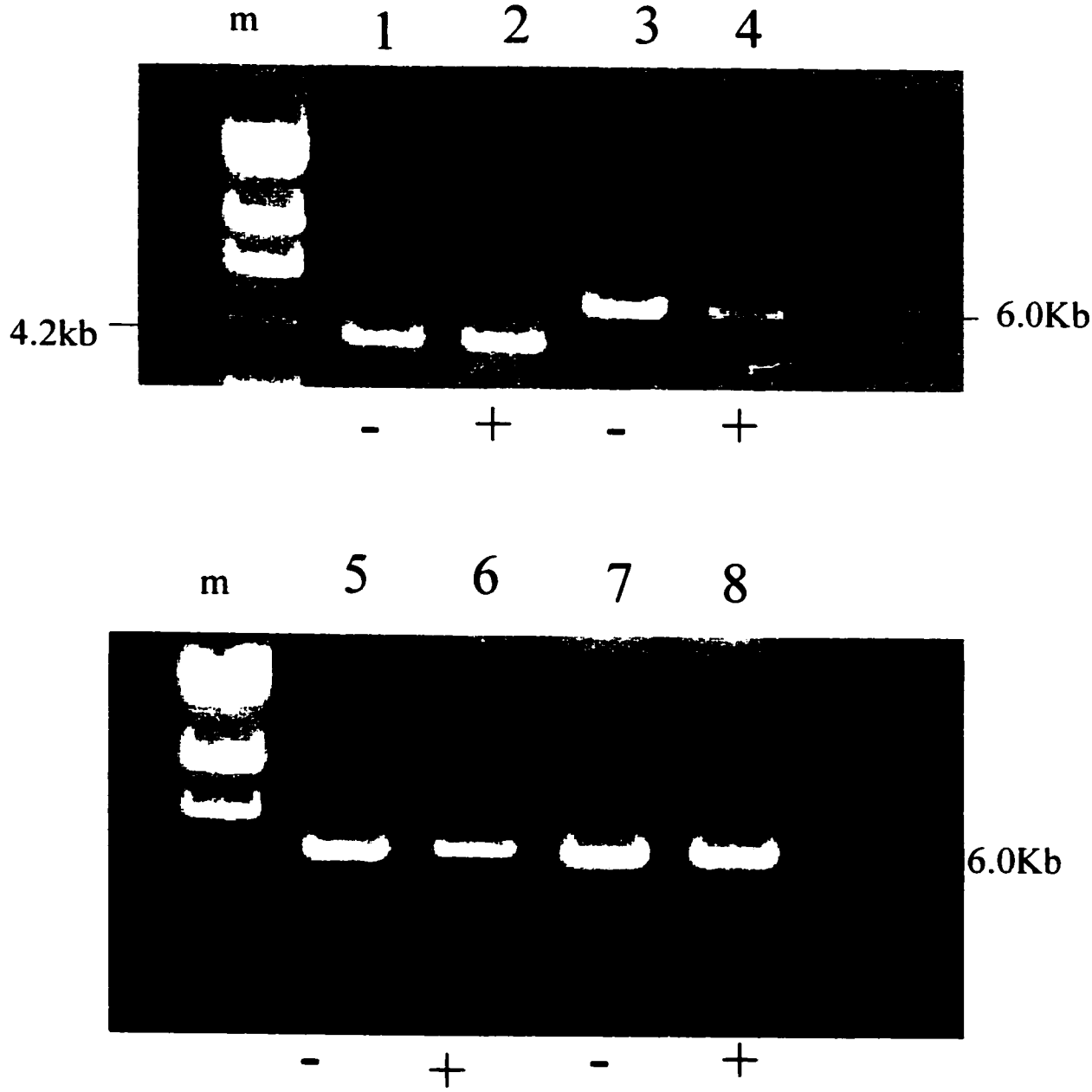


Figure 27. Effect of 5-azacytidine on DNA plasmid replication in the presence of mutant methylases. Plasmid DNA preparations from CC221 cells grown to the same OD_{600} were electrophoresed in a 1% agarose gel. Lanes labeled m are the λ HindIII molecular weight markers. Lanes 1 and 2 are the empty plasmid pACYC184 untreated and treated with 10μg/mlof 5-azacytidine respectively. Lanes 3 and 4 are plasmid pDV101 untreated and treated with 10 μg/ml of 5-AC respectively. Lanes 5 and 6 are the pDV105 mutant ser177 *dcm* plasmid untreated and treated with 10 μg/ml of 5-AC respectively. Finally, lanes 7 and 8 are the mutant trp177 *dcm* Plasmid untreated and treated with 10 μg/ml of 5-AC respectively.

3.4.3. Growth curve of cells containing wildtype and mutant methylases

CC221 cells, devoid of the *dcm* gene, were provided with fresh media, with or without 10 μg/ml of freshly prepared 5-azacytidine, and were grown with aeration at 37 degrees Celsius. Time points taken are depicted in Figure 28. There is a slight decrease in cell growth with 5-azacytidine treated wildtype cells containing the control plasmid. This same small decrease is seen with the plasmid (pDV107) containing the gene coding for the tryptophan mutant. This is evidence that binding of the methylase to the DNA is necessary for the effect on cell growth or cell division. Cells harboring the plasmid that expresses the wildype methylase (pDV101) and treated with the drug grow much more slowly than the untreated pDV101-containing cells by as much as 3.5 fold and do not reach the same saturation levels after 24 hours. The treated cells containing the plasmid with the gene for the mutant methylase (pDV105) also grow more slowly than their untreated counterparts, but not as slowly as treated cells transformed with pDV101. There is as much as a 1.75 fold decrease in these cells, expressing the mutant pDV105, compared to the untreated cells. These results support the experiments showing that in both cases the wildtype Dcm has the greatest effect on plasmid yield in cells treated with 5AC.

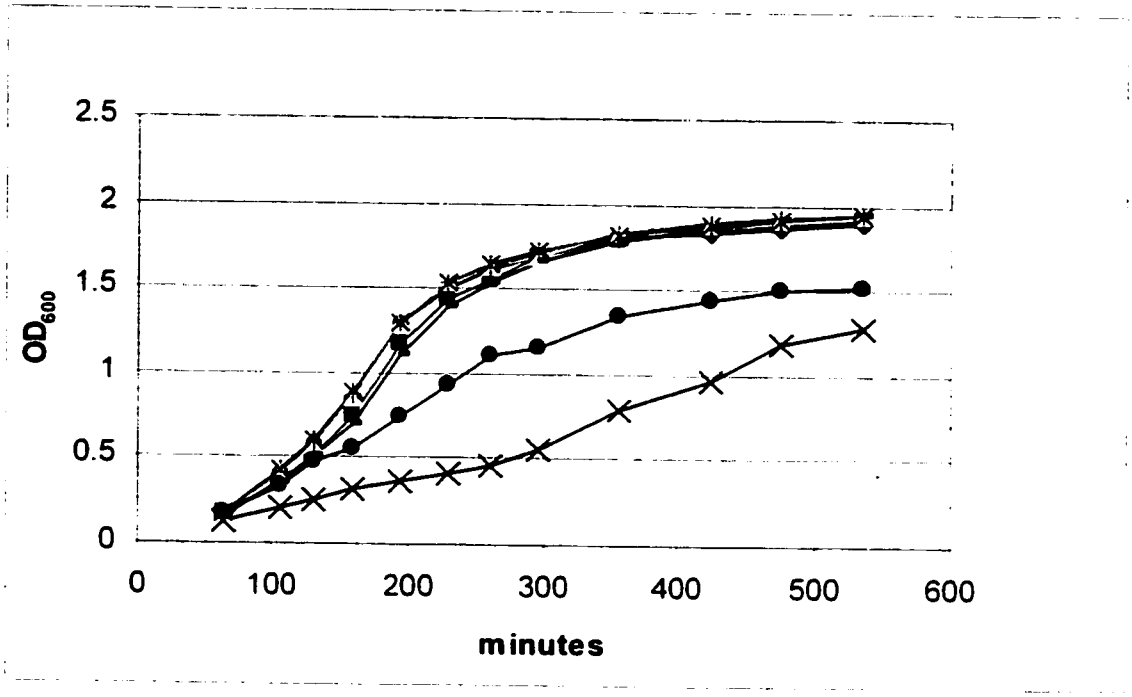


Figure 28. Growth curve for 5-azacytidine treated cells containing various plasmids. CC221 containing pDV101, treated (✕) and untreated (▲),pDV105, treated (●) and untreated (✱) and pDV107, treated (-) and untreated (+). The concentration of 5-azacytidine used for the treated samples is 10 μg/ml.

3.4.4. Cells treated with 5-AC in the presence of over-expressed dcm elongate

CC221 cells treated with 5-azacytidine and containing the over-expressed wildtype *dcm* methylase, filament as shown in Figure 29, panel 4, whereas cells missing the over-expressed methylase or the drug do not (Figure29 panel 2 and 3). Similarly to the plasmid DNA experiments and growth effects mentioned above, the binding of the methylase is necessary for the filaments to appear. Mutant C177S (Figure 30 panel 2) causes cells to filament to an even greater extent than the wildtype, and the C177W mutant Dcm does not cause cell filamentation (Figure 30 panel 4). The 5-azacytidine Dcm covalent lesions are, therefore, the probable cause of these effects on the cell and its DNA.

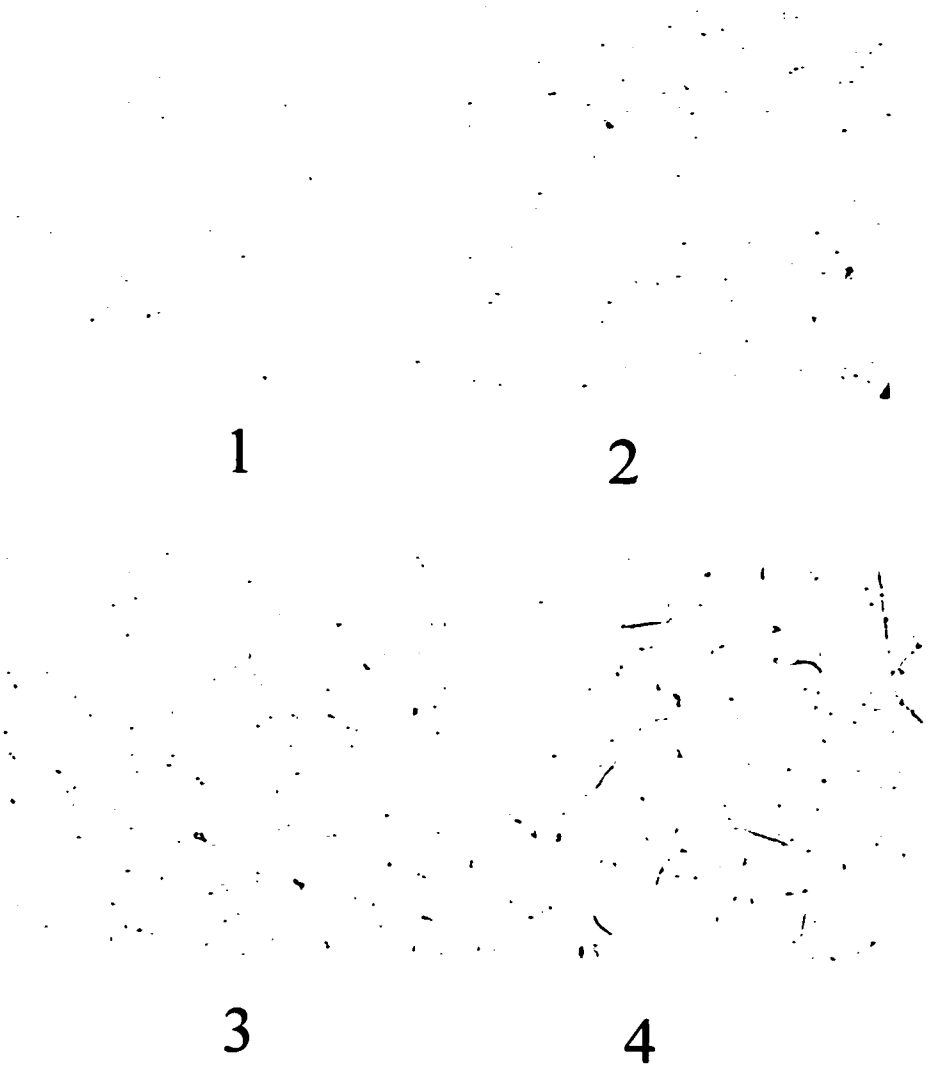


Figure 29. 5-azacytidine induced cell elongation.
Panel 1 and 3 are untreated CC221 cells containing pACYC184 and pDV101 plasmids respectively. Panel 2 and 4 are CC221 cells treated with 10 μg/ml of 5-AC and containing pACYC184 and pDV101 respectively. Cells shown in this figure were stained with methylene blue.

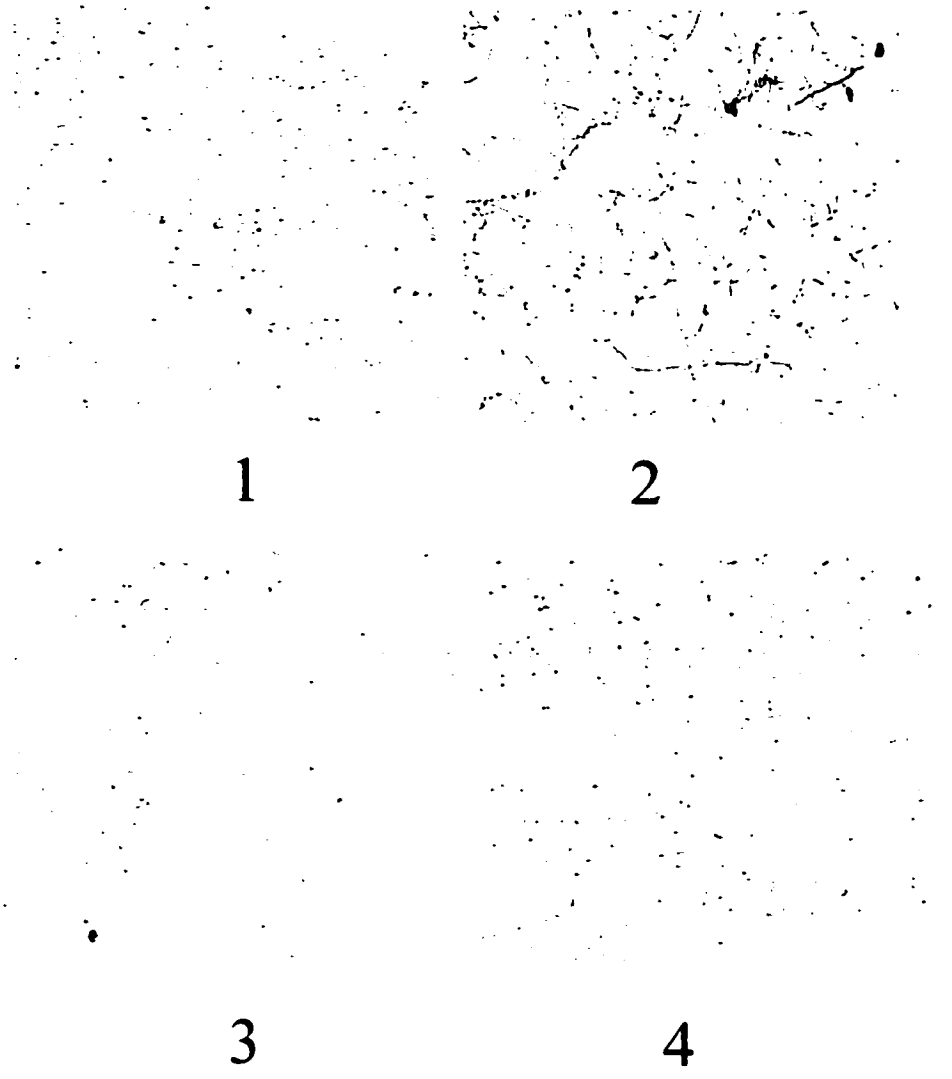


Figure 30. Effect of 5-AC on cell elongation in the presence of mutant methylases. Panel 1 and 3 are untreated CC221 cells containing pDV105 and pDV107 plasmids respectively. Panel 2 and 4 are CC221 cells treated with 10 μg/ml of 5-AC and containing pDV105 and pDV107 respectively. Cells shown in this figure were stained with methylene blue.

3.4.5. DNA replication blocks

To detect any replication blocks that may be caused by the Dcm dependent 5-azacytosine lesion, the plasmid replication fork was visualized using two-dimensional agarose gels. Figure 31, panel 1, shows the replication fork of the DNA from cells containing a plasmid expressing the wildtype *dcm* gene. Figure 31, panel 2, displays the replication fork from the same cells but this time treated with 10 μg/ml of 5-azacytidine. The treated cells with Dcm lead to blocks in the replication fork depicted by the arrows in the figure.

Figure 32 show the results for the replication forks for plasmids which were prepared from cells which contained mutant methylases C177S and C177W. Similarily, the plasmids from cells containing the mutant methylases C177S and C177W treated with 10 μg/ml of 5-azacytidine are shown in Figure 32 (panel 1 and 2) and Figure 32 (panel 3 and 4) respectively. There is a block present in plasmids carrying the C177S mutant *dcm* gene in the 5-AC treated sample but not in plasmids with the C177W mutant gene treated with 5-AC. These results complement the results of the decrease in DNA replication as well as the filamentation results since the block in replication can lead to inhibition of cell division leading to elongated cells.

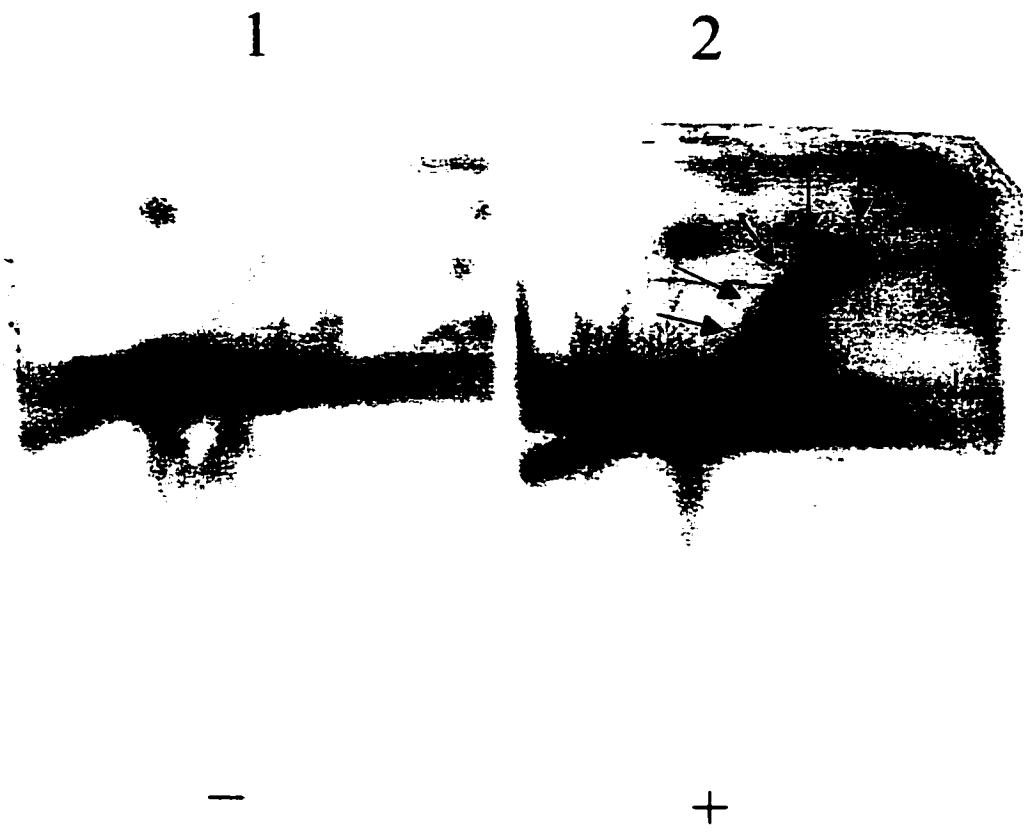


Figure 31. 5-azacytidine induced blocks in DNA replication forks.
Southern blots for 2-D gels. Panel 1 is pDV101 plasmid DNA.
CC221 cells used to isolate this plasmid were untreated. Panel 2 is pDV101 plasmid from CC221 treated cells with 10μg/ml of 5-azacytidine.

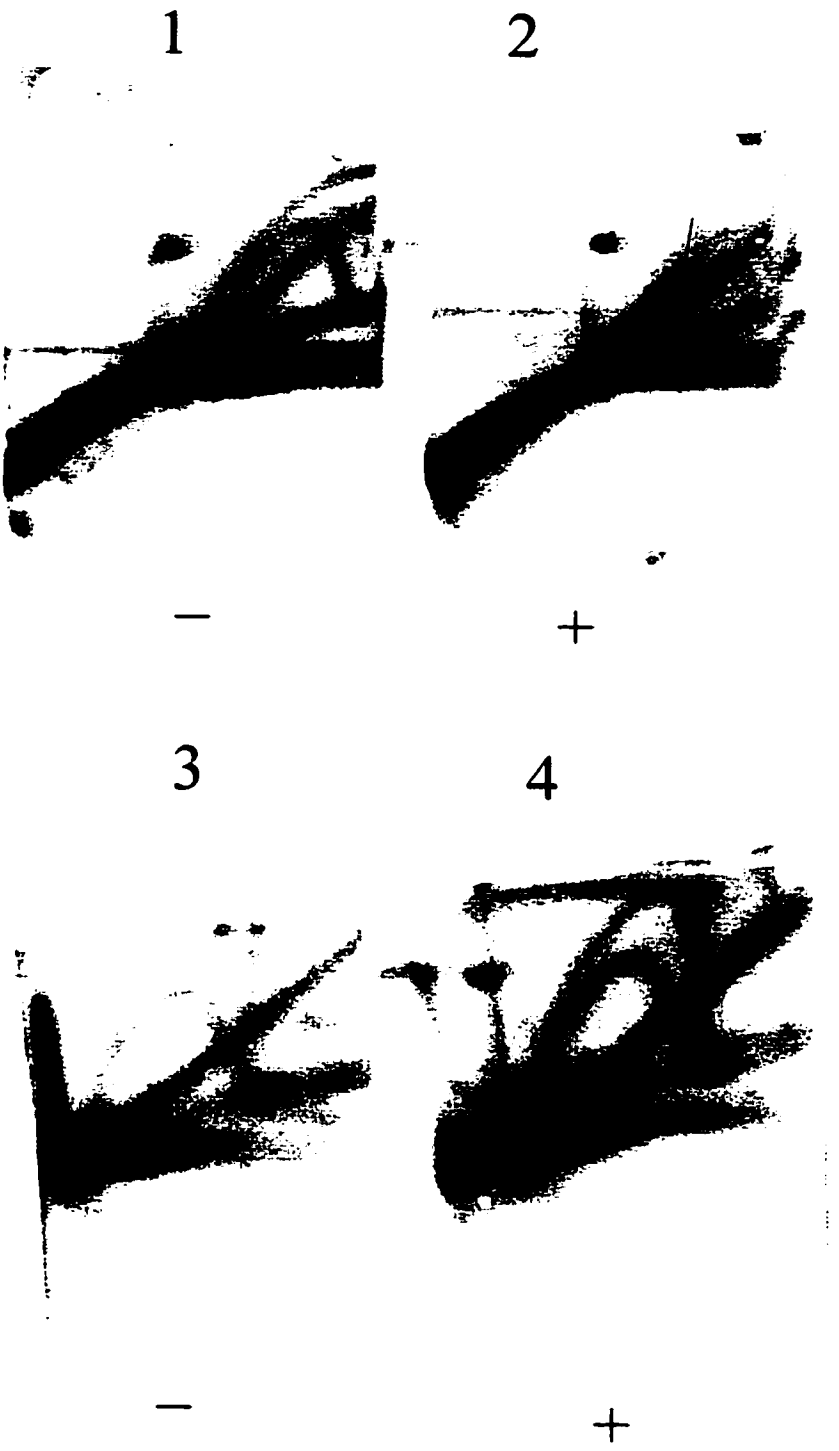


Figure 32. Effect of 5-AC on blocks in DNA replication in the presence of mutant methyltransferase. Southern blots for 2-D gels. Panel 1 is pDV105 plasmid DNA from untreated cells. Panel 2 is pDV105 plasmid from CC221 cells treated with 10μg/ml of 5-AC. Panel 3 and 4 are pDV107 plasmid from untreated and treated cells, with 10μg/ml of 5-azacytidine, respectively.

4. DISCUSSION

4.1. Regulation of *vsr* and *dcm*

We have shown that the Vsr endonuclease is growth phase regulated since Western blot analysis reveals low levels of Vsr protein in early log and log phase cells, and higher levels in late log and stationary phase cells (Macintyre *et al.*, 1999). However, Dcm was shown to be independent of growth phase since its levels were consistant thoughout the growth phase. Previous studies showed that Vsr actually disappears very rapidly when staionary-phase cells are diluted into fresh medium, suggesting that the protein is actively degraded as cells prepare to re-enter log phase (Macintyre *et al.*, 1999). Also, the pattern of reduced expression during log phase is maintained even in cells expressing only *vsr* from a synthetic promoter. Therefore, the fact that the dcm promoter is not required for the growth phase-dependent regulation suggests that a large part of the regulation is post-transcriptional. Also, operon structure regulation was also observed since the levels of Vsr were higher when expressed in the absence of the *dcm* overlapping gene. One reason may be that in some instances, the *vsr* ribosome binding site may be inaccessible to the translational machinery for Vsr due to ongoing Dcm translation.

It is not yet known what mechanism controls growth phase-dependent production of Vsr. The targeted proteolysis of *E.coli* sigma factor, σ^S, during log phase and its stabilization during stationary phase provided an attractive model for this form of regulation (Zhou and Gottesman, 1998). Various protease deficient strains were used, including the *clpP* deficient strain presented in this study. Other strains tested by researchers in our lab included *rssB*, *lon*, and a *lon clpP* double mutant (unpublished

data). However, none of the proteases known to act on the sigma factor were found to affect the growth phase regulation (Figure 8 and other data not shown). A library screen or proteomic approach might be better at identifying the proteins involved in Vsr post-transcriptional regulation.

Based on a previous observation that high levels of Vsr are mutagenic (Doiron *et al.*, 1996), it was hypothesized that levels of the Vsr endonuclease are tightly controlled in growing cells (Macintyre *et al.*, 1997). In particular, mutagenesis could be avoided by keeping levels of Vsr low during periods of DNA replication in order to avoid interference with mismatch repair. Also, the limited amounts of Vsr available in log phase to repair deamination damage could be a major contributor of 5-methylcytosine mutability in *E.coli*.

4.2. 2-aminopurine induced mutagenesis

2-aminopurine causes A-C mismatches which have been postulated to be subject to mismatch repair. However, in certain sequence contexts there may be other factors which change the dynamics and the accessibility of that lesion. For example, CC(A/T)GG sequences are hotspots for 2-AP mutagenesis in the *lacI* gene (Coulondre *et al.*, 1978b).

4.2.1. 2-aminopurine induced transition mutations are due in part to Dcm

We have examined the effect of the cytosine methylase, Dcm, on 2-aminopurine induced transition mutations. We have shown that when the methylase is present, 2-AP induced C-to-T and T-to-C mutations occur at lower doses of 2-AP than in cells without the *dcm* gene. Also, for the C-to-T mutations, this increase is greater than that which

would be accounted for by deamination events from a methylcytosine to a thymine. This result is also further supported by the fact that adding back the *vsr* gene to cells treated with 2-AP leads to an increase in C-to-T mutations which are as great as those in cells with only the *dcm* gene minus the deamination effect. Therefore, Dcm does contribute to 2-aminopurine induced transition mutations.

Furthermore, it has been suggested in previous studies that 2-aminopurine saturates the mismatch repair system leading to increases in frameshift and transition mutations (Macintyre *et al.*, 1997). However, our results suggest that the transition mutations caused by 2-AP are not due to saturation of the MMR system. Evidence for this was that the addition of MMR proteins did not reduce the 2-AP induced mutations. Also, Dcm was found not to block 2-AP-C lesions since in a *dcm* deficient strain with the addition of MMR proteins, only the pMQ339 plasmid expressing the MutL protein caused a small decrease in mutations.

4.2.2. Mismatch repair and 2-aminopurine at methylase recognition sites

Our results also support the hypothesis that MMR does not target 2-AP-C lesions since changes in MMR protein levels had no effect on 2-AP induced transitions in either dcm^+ or dcm^- strains as mentioned above. Further evidence for this hypothesis are the results shown with the mismatch repair deficient strains. No additional increase in mutations was noted with increasing doses of 2-AP in these deficient strains compared to cells proficient in MMR. The only differences noted with these strains is a small increase which was observed due to background in untreated cells representing a slight saturation of MMR at that site.

The effect noted with Dcm and 2-AP induced transitions could be due to the methylation which increases the ability of 2-AP to mispair with C. Evidence for this hypothesis includes data which show that a mutant methylase, unlike the wildtype, does not increase 2-AP-stimulated mutations (Figure 14). This mutant methylase C177W, also used in 5-azacytidine experiments, is expected to bind but not methylate the sequence.

4.3. 5-azacytidine induced Dcm independent lesions

This study has demonstrated that the lesion caused by the 5-azacytidine drug is a target for elements of both mismatch and nucleotide excision repair. The evidence for this conclusion is presented here.

4.3.1. 5-AC causes C-to-G transversion mutations via C-C mispairs

C→G transversion mutations arise via either a G-G mismatch or a C-C mispair; however, G-G lesions are good substrates for the mismatch repair system (Su *et al.*, 1988). Mutants deficient in this repair system should all display an increase in C→G mutations. This was not the case. No increase was detected for the *mutH* strain. In addition, 5-azacytidine is a cytidine analog which presumably gets incorporated into the DNA as 5-azacytosine as has been shown *in vitro* (Friedman, 1985). This makes the C-C lesion the most probable. Moreover, C-C mismatches have recently been shown to be corrected in *S. pombe* by nucleotide excision repair and since C-C mispairs are poor substrates for the mismatch repair system another system must be involved. The results from Figure 17 show that, in fact, an increase in mutation frequency did occur in the nucleotide excision deficient strains, *uvrA*, *uvrB* and *uvrC*. These results suggest that the

NER system is the primary system involved in the repair of theses 5-AC lesions. Therefore, like *S.pombe*, *E.coli* could also repair its C-C lesions through its NER system.

4.3.2. Genetic evidence for interactions between MMR and NER

The increase seen with the *mutL*⁻ and *mutS*⁻ strains implicates elements of the mismatch repair system as an important player in the correction of 5-azacytidine-induced lesions. The differences in the various single mutants give rise to information about the mechanism involved with such repair. The fact that the *mutH* mutant does not increase the number of C→G mutations indicates that mismatch repair is not the prime repair system for the 5-azacytidine-induced lesions since all components of the repair system should have increased the mutation rate and this was not the case. In addition, the *mutH* strain actually reduced these mutations. Perhaps its association with the other MMR proteins inhibits the remaining partners, MutL and MutS, from acting in an alternative pathway.

Furthermore, the increase depicted by the *mutS* and *mutL* mutants was not as dramatic as that seen by the *uvrA*, *uvrB* and *uvrC* mutants, which may help to elucidate the mechanism at hand. This difference may imply that the MutS and/or MutL proteins actually are involved in stimulating the endonuclease activity of UvrB and UvrC.

The proposed mechanism presented here is plausible based on previous studies. Both MutL and MutS, or homologues there of, have been reported to be involved in a number of different pathways including recombination repair (Friedberg *et al.*, 1995), transcription coupled nucleotide excision repair (Mellon and Champe, 1996), nucleotide excision repair in yeast (Bertrand *et al.*, 1998), and apoptosis (Li, 1999).

4.3.3. Physical interactions between the MMR and NER proteins

Using the bacterial two-hybrid system as a tool to look at the interactions between MMR proteins and NER proteins proved to be quite informative. Treating cells with 5-azacytidine ensured that the lesion under investigation was present. The results lead to the discovery of the interaction of UvrB and MutL, as well as, UvrB and MutS. These interactions corroborate the genetic data which also were shown in the presence of the drug. In addition to these drug-dependent interactions, a novel interaction between UvrC and MutL was also noted, and this was independent of the presence of 5-azacytidine.

Perhaps there is an alternative or hybrid repair system that involves components of both the mismatch repair system and the nucleotide repair system. A model involving either UvrA and/or MutS as a recognition protein, the MutL as the molecular matchmaker and the two endonucleases, UvrB and UvrC for the removal of the patch could work together as the alternative repair system. However, since MutS has not been shown to be involved in the repair of C/C mispairs (Su *et al.*, 1988) the mechanism could involve MutS and MutL in NER. MutS and MutL could act as stimulatory factors. It also is also possible that not all the players involved have been discovered and that there may be parallel pathways that recognize the same lesions. For *E.coli* these could include MutM, since a recent publication has shown that the MutM purified glycoslyase binds to a C/C containing heteroduplex (Nakahara *et al.*, 2000). Also, another likely pathway is recombinational repair. The extreme sensitivity of *recA* mutants to 5-AC supports this.

No UvrA-UvrA dimerization , UvrA-UvrB, or UvrB-UvrA interactions were noted (data not shown). This may be due to the conformation of some of the fusion proteins which may not allow both N-terminals of the UvrA proteins to interact and a trimeric protein with the UvrB protein to follow because of how they are cloned.

Furthermore, the heterogeneity of the UvrB and MutL, and UvrB and MutS interactions still remains unexplained. However, there is the possibility that the heterogeneity is due to the random incorporation of the drug into the cells and/or DNA at various sites. Another possibility is that SOS induction is needed since *uvrB* is SOS-inducible whereas uvrC is not (Friedberg *et al.*, 1995). Also, once again the cloning could be the reason since they are fusion proteins and the terminal domains involved with the specific interaction may not be completely accessible.

The heterogeneity associated with the interactions seen are not due to random mutations caused by the drug. Cells containing the pT18 and pT25 empty bacterial two-hybrid vectors, treated with the same concentration of 5-azacytidine, were plated onto rifampicin containing plates. These cells lead to the same number of rifampicin resistant colonies as those containing the bacterial two-hybrid proteins which showed interactions (data not shown).

The interaction results presented in this section support published data of interactions between the mismatch repair and the nucleotide excision repair systems previously seen in other organisms such as *Saccharomyces cerevisiae* (Bertrand *et al.*, 1998), and humans (Mellon *et al.*, 1996) (Fishel, personal communication). *E.coli* has simpler repair systems and, therefore, acts as a good model system. Forming repair complexes between systems allows *E.coli*, and other organisms, to provide for the

recognition of many more lesions and in a more specialized manner, perhaps even for new foreign lesions such as those incorporated by a drug such as 5-azacytidine. The new discovery of the overlaps between the various repair systems opens new doors to the possibility of elucidating mechanisms for established repair systems and gaining further knowledge on these new repair complexes.

4.3.4. 5-AC induced frameshift mutations are not due to polymerase IV, Dcm covalent complexes, or Vsr

The results of this study indicate that the frameshift mutations caused by the drug do not support the idea of a simple saturation of mismatch repair proteins. This lack of saturation is also consistent with our findings that MMR is not the system involved in preventing 5-AC stimulated mutations. Nor are the increases due to SOS-induction of error-prone DNA polymerase. Furthermore, it was also shown that these frameshift mutations are not a result of the covalent complex formed by the 5-azacytosine containing DNA with the cell's methylase, Dcm. The data presented using a *dcm* deficient strain showed that the number of frameshifts was not reduced.

It is possible that 5-azacytidine is causing these frameshift mutations directly, perhaps in a similar fashion as the C-to-G's albeit to a lesser extent. The mechanism here would involve an extra guanine being incorporated opposite a run of cytosines that may have randomly incorporated a 5-azacytosine making it more susceptible to the error by the DNA polymerase.

4.4. 5-azacytidine induced Dcm dependent lesions

5-azacytidine has previously been shown to cause covalent complexes with methyltransferases (Friedman and Som, 1993). We have investigated the effects of these covalent complexes that arise when 5-azacytosine-containing DNA is bound to the methylase, Dcm.

4.4.1. 5-azacytidine's effect on DNA replication and cell growth

We show that the presence of the Dcm protein in cells which have been treated with 5-azacytidine causes decrease in plasmid DNA replication as well as an increase in cell filamentation, both of which indicate an inhibition of DNA replication (Elwell *et al.*, 1987). Furthermore, cells treated with the drug and expressing *dcm* also grow at a reduced rate and do not reach the same saturated density that untreated cells do. The decrease in DNA replication was confirmed by replication fork analysis whereby blocks in the fork are seen.

4.4.2. Mutant Dcm methyltransferases

The results from the mutant methylases helped define the mechanism leading to the reduced DNA replication and inhibition of cell division. The binding of the methylase to the 5-azacytosine-containing DNA is crucial for the effect. The use of a mutant Dcm methylase, C177S, which binds to the DNA containing 5-azacytosine but does not methylate it, showed that the binding process was, in fact, necessary for the reduced

DNA replication and the inhibited cell division. This supports the hypothesis that the covalent complex lesion is inhibiting the replication machinery. Similarily, the Dcm mutant, C177W, which neither binds nor methylates was shown not to cause any lesions, and did not block DNA replication or division.

The C177S mutant however, does not respond like the wildtype. It is not as sensitive to the drug as seen by the growth curve. It also does not lead to as much of a decrease in plasmid DNA replication. C177W does not bind to regular cytosines (Doiron *et al.*, 1999;Wyszynski *et al.*, 1992) but does bind to DNA containing 5-azacytosine in the CCA/TGG sequence. However, the results could indicate that this mutant does not bind as well or as irreversibly as the wildtype Dcm leading to the reduced effect since the amount of mutant methylase made in a cell is comparable to the wildype methylase (Figure 25) (Doiron *et al.*, 1999).

The Dcm dependent lesions must be repaired since cells treated with the drug and containing the methylase are viable, but there is a small decrease in the cell growth and they do not reach the same cell density. However, the treated cells which over-express the methylase display a dramatic drop in cell growth and also reach a cell density which is 3.7 fold less than the wildype. The repair of these lesions is probably performed by the recombinational repair system. The evidence for this is first, that cells deficient in the *recA* gene are extremely sensitive to the drug (Bhagwat and Roberts, 1987)(Cupples unpublished results) and second, there is evidence that cells with no methylase, but which are still *recA* deficient are 2-fold less sensitive to 5-azacytidine (Bhagwat and Roberts, 1987). Although there is still a sensitivity to the drug for other reasons the

reported decrease in sensitivity could indicate that the repair of these lesions are at least due, in part to repair by recombination.

Put together, these results emphasize that methylation and methylases play an important role in both spontaneous and mutagen-induced mutations. Also, we have shown that these mutagens have served as powerful tools in the analysis of DNA repair processes. The balance of repair systems in the presence of methylatable and non-methylatable sequences containing spontaneous and chemically-induced mispairs is crucial in maintaining genome integrity.

5. CONCLUSIONS

1. Vsr is post-translationally regulated.
2. Operon structure contributes to decreased production of Vsr.
3. ClpP is not the protease responsible for the post-translational regulation of Vsr.
4. Dcm does not block access of MMR to methylation sites containing 2-AP induced A-C lesions.
5. C/A mismatches caused by 2-aminopurine within the methylase recognition sequence are not reduced by increased amounts of MMR proteins.
6. 5-AC stimulated C-to-G mutations in *E.coli* are affected by mutations in both MMR and NER genes. NER is the main repair system involved in the repair of the 5-AC independent lesions. MutL and MutS are also needed.
7. 5-azacytidine induces frameshift mutations which are not due to pol IV, Dcm complexes, or Vsr levels but may be due to increased DNA polymerase slippage at 5-AC in runs of C's.
8. 5-AC dependent interactions between the repair proteins UvrB and MutL, and UvrB and MutS were observed with the bacterial 2-hybrid system. Using the same system, interactions between UvrC and MutL were also observed.
9. 5-azacytidine induced Dcm lesions cause replication blocks and lead to a decrease in DNA replication.
10. *E.coli* cells treated with 5-AC and over-expressing *dcm* lead to a decrease in cell growth and a block in cell division represented by long filaments.
11. Dcm dependent 5-azacytidine-induced effects are observed with the mutant Dcm C177S, but not with the mutant Dcm C177W

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