

An Abstract of the Thesis of

Timothy J. Brodeur for the degree of Bachelor of Science in Bioresource Research: Biotechnology, presented on June 13, 2000. Title: DNA Mismatch Repair and O⁶-Methylguanine Toxicity: Testing the Futile Repair Cycle Hypothesis.

Cell lines deficient in certain genes of the DNA mismatch repair (MMR) system show a marked resistance to the cell killing or apoptotic effects of DNA alkylating agents. The molecular mechanism for this resistance is not yet understood, but is currently of great interest because of the recent use of these agents in cancer chemotherapy. Of particular interest is the role of the primary mutagenic base-adduct, O⁶-methylguanine (O⁶-meG), in this MMR-dependent resistance. In this paper, an assay for testing one of the hypotheses for the MMR-dependent resistance to alkylating agents (the futile repair cycle) is described and the progress towards performing such an assay is reported. Specifically, single-stranded phage DNA was prepared with less than 5% double-stranded DNA contaminant, fl phage gene II-protein was purified with a specific activity of approximately 5% of the published value using an abbreviated protocol, and MMR-competent HeLa cell nuclear extract was prepared with an *in vitro* repair efficiency of 30%. Also, the possibility of improving *in vitro* repair assays by using alkaline phosphatase in DNA substrate preparation is investigated.

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**DNA Mismatch Repair and O⁶-Methylguanine Toxicity:
Testing the Futile Repair Cycle Hypothesis**

by

Timothy J. Brodeur

A Thesis Submitted to
Oregon State University

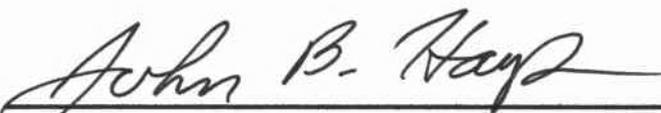
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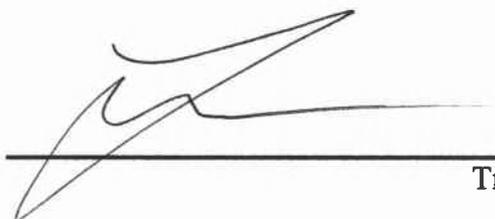
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Timothy J. Brodeur, Author

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Introduction

DNA Mismatch Repair: Background

Mismatched bases in DNA are a relatively common occurrence in all organisms⁽¹⁾. Most mispairs arise from replication errors, but can also come about through chemical processes such as deamination of bases, or genetic processes such as recombination of homologous DNA regions. Regardless of origin, mispairs pose a risk for generating potentially deleterious mutations. Not surprisingly, both prokaryotic and eukaryotic organisms possess a repair pathway for correcting mispairs in DNA⁽²⁾. This system, referred to as the DNA mismatch repair (MMR) system, has been extensively studied over the last decade and many of the molecular details have been well characterized.

MMR is an enzyme system that has been highly conserved throughout evolution⁽³⁾. It is extremely important for maintaining genetic stability, with MMR-deficient cell lines showing spontaneous mutation rates 1000-fold higher than wild-type. The primary substrate for MMR is single base/base mismatches (G/T, A/C, G/G, C/C, etc.)^(4,5). Each type of mismatch is corrected by MMR at a different efficiency, with G/T mispairs being the best substrate and C/C being the worst substrate. Also corrected by MMR are small loop-outs in DNA arising from template slippage during replication. Loop-outs generally occur in highly repetitive regions of DNA such as mono-, di-, or trinucleotide repeats. Unrepaired, loop-outs can result in insertion or deletion mutations.

MMR functions via a so-called, long-patch repair pathway, with excision of the entire region of DNA containing the error⁽²⁾. Specific details vary between prokaryotes and eukaryotes, but a general mechanism for MMR can be described as follows⁽⁴⁾:

1) recognition of a mismatch or loop-out in DNA, 2) nicking of the newly synthesized strand or recognition of a 3' end in this strand, 3) removal of the "incorrect" region of the new strand, 4) subsequent resynthesis. Excision can proceed bidirectionally from the mismatch, and is currently believed to involve non-MMR-specific exonucleases.

Resynthesis is performed by general replicative polymerases.

In prokaryotes, the MMR system consists of three proteins: MutS, MutL, and MutH^(4,5). In eukaryotes, several proteins homologs of both MutS and MutL have been identified. No MutH homolog has yet been identified.

MutS and its homologs are believed to be the main initiating factor of MMR, binding the mispair and recruiting the other MMR components^(4,5). Heterodimers of MutS homologs bind mismatches in eukaryotic systems. MSH2 and MSH6 form a heterodimer referred to as MutS α , which shows a high affinity for single base-base mismatches and small loop-outs. A second heterodimer, MutS β , is formed by MSH2 and MSH3. This complex shows affinity for loop-outs specifically.

The roles of MutL and its homologs are not completely understood, but are essential for fully functional MMR^(4,5,6,7). Analysis of the bacterial MutL crystal structure indicates that it may belong to the ATPase class of enzymes and that it has structural homology to certain molecular chaperones⁽⁷⁾. Prokaryotic MutL is known to form a stable complex with MutS and also interacts with MutH, stimulating the endonucleolytic

activity of this protein (discussed below)^(5,6). As with MutS, eukaryotic MutL homologs function as heterodimers^(5,6).

MutH is an endonuclease that specifically introduces single-stranded nicks at unmethylated GATC sites in hemimethylated DNA^(4,6). This activity is stimulated by MutL and initiates strand excision of the error-containing strand. As mentioned previously, no eukaryotic homolog of MutH has yet been observed, and it is not understood how strand differentiation occurs in eukaryotes⁽⁸⁾. Providing a nicked-DNA substrate containing mismatches to MMR-competent eukaryotic cell extracts will, however, result in excision and repair of the nicked strand⁽⁹⁾.

An important development in the study of MMR has been the discovery that certain forms of human cancer seem to involve a breakdown in the MMR system. Tumor cell lines isolated from hereditary non-polyposis colon cancer (HNPCC) show a marked defect in certain MMR genes and are characterized by microsatellite instability (large variations in repetitive DNA regions consistent with a non-functional MMR system)^(4,5). Genetic analysis of HNPCC patients also shows a tendency for a defect in one allele of the MMR genes *hMSH2*, *hMLH1*, or *hPMS2* in non-tumor tissue, and a subsequent mutation in the second allele in tumor tissue. These findings, and other tumor development research, seem to imply that MMR and cancer predisposition are closely linked. As will be discussed, this may have serious implications for the use and development of certain chemotherapy agents.

DNA Alkylating Agents and O⁶-Methylguanine

In addition to deamination reactions, methylation can also yield mismatches in DNA⁽¹⁰⁾. In fact, DNA alkylating agents such as N-methyl-N'-nitrosoguanidine (MNNG) and N-methyl-N'-nitrosourea (MNU) have long been used in microbiology and molecular biology to promote mutagenesis. These agents react with DNA to form O-alkylated and N-alkylated base adducts. One of the primary modified bases is O⁶-methylguanine (O⁶-meG).

The mutagenic effect of O⁶-meG residues in DNA is manifested in a G→A transition mutation following semiconservative replication. Crystallographic studies have shown that O⁶-meG is capable of forming stable base-pairs with both thymine (in standard Watson-Crick conformation) or cytosine (in wobble conformation) (Figure 1)⁽¹¹⁾. Although these base pairs are thought to be approximately equal in stability, studies have suggested that DNA polymerase reads O⁶-meG as an adenine residue in the template strand and incorporates a thymine, leading to the high mutation rate observed in methylated DNA⁽¹²⁾.

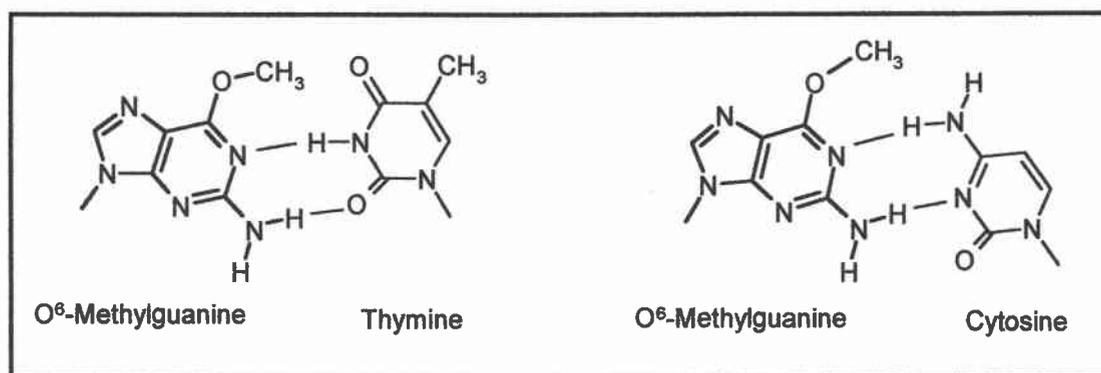


Figure 1: Base-Pairing Properties of O⁶-Methylguanine. Crystallographic studies have demonstrated that O⁶-meG residues form stable base-pairs with both thymine (in standard Watson-Crick conformation) and cytosine (in wobble conformation).

To avoid these potentially harmful mutations, a specific repair pathway exists for correcting O⁶-meG residues in DNA in both prokaryotic and eukaryotic organisms⁽¹⁰⁾. This pathway involves alkyltransferase enzymes that directly remove the methyl-group from O⁶-meG residues. Alkyltransferases are a class of proteins known as suicide enzymes. Such enzymes form covalent bonds with their substrate and are thus consumed in the reactions they catalyze. Cell lines defective in alkyltransferase genes show increased mutation rates when exposed to DNA alkylating agents. Studies of eukaryotic cell lines have also demonstrated an increased ability to induce tumors by treatment with alkylating agents⁽¹⁰⁾.

In addition to their mutagenic effects, DNA alkylating agents also have a cytotoxic action⁽¹⁰⁾. Treatment with large doses of alkylating agents causes cell killing, particularly in eukaryotic cell lines where hypermethylation triggers apoptosis or cell suicide. This effect has been utilized in the development of chemotherapeutic agents in cancer treatment, the most notable of which is temozolamide or TMZ⁽¹³⁾. Originally investigated for use in treating melanoma, TMZ has recently been granted accelerated approval for treatment of malignant brain tumors⁽¹⁴⁾. Interestingly, it was discovered that the mutagenic and cytotoxic effects of alkylating agents could be uncoupled and that this uncoupling is MMR-dependent⁽¹⁰⁾. Studies have shown that MMR-deficient cell lines display a marked resistance to the apoptotic effects of alkylating agents while still undergoing the mutagenic effects of these chemicals^(10,13).

Observations such as these have serious biological implications with regard to the future use and development of alkylating agents in chemotherapy^(13,15). Not only will certain tumor cells not be affected by these agents, this apoptotic resistance may

effectively act to select for MMR-deficient cells in the patients being treated. Since such cells are cancer prone, treatment with alkylating agents presents the risk of giving rise to secondary cancers in non-tumor cells. This concern has prompted much research to understand the exact mechanism for this resistance.

DNA Mismatch Repair and O⁶-Methylguanine: The Futile Repair Cycle

One of the initial studies to critically address the involvement of MMR in the apoptotic response to alkylating agents was conducted by D'Atri, *et al.* in 1998⁽¹³⁾. In this study, the authors compared the effects of TMZ on a methyltransferase-deficient tumor cell line, TK6, with that of an MMR-deficient derivative of this line, MT1. MT1 possesses a mutation in the *MSH6* gene making it incapable of forming the MutS α complex. As a control, the agent etoposide (a topoisomerase inhibitor that induces apoptosis) was used to assess alkylation-independent apoptosis of each cell line.

The study found that while TK6 cell growth dramatically decreased with increasing doses of TMZ, similar MT1 cultures showed no significant change in cell number. Cytometric analysis showed a cell cycle shift characteristic of apoptosis in TMZ treated TK6 cultures. Treated and untreated MT1 cultures displayed no distinguishable difference in cell cycle profile. Lastly, while TMZ treatment induced phosphorylation of p53 (a signal protein implicated in the apoptotic response) in TK6, no such response was observed for MT1 cells. All assays were performed in parallel with etoposide and no difference was noted between the apoptotic responses of TK6 and MT1 cells.

Another studies has demonstrated that both O⁶-meG/T and O⁶-meG/C base-pairs are bound by MutS α ⁽¹⁶⁾. Additionally, it was shown that O⁶-meG-containing DNA

substrate initiates an MMR-response *in vitro*⁽¹⁷⁾. These observations have led to a proposed model for the apoptotic response to O⁶-meG residues in DNA, referred to as the futile repair cycle hypothesis⁽¹⁰⁾. This model states that an O⁶-MeG residue present on the original or template strand will still be present after the first round of repair, and the MMR system will then initiate a second round of repair. It is proposed that this futile cycling would result in persistent single-strand DNA breaks which in turn would yield double-strand DNA breaks following a second round of replication, triggering cell-cycle arrest and programmed cell death (Figure 2). However, experiments explicitly testing the futile repair cycle model have not yet been conducted.

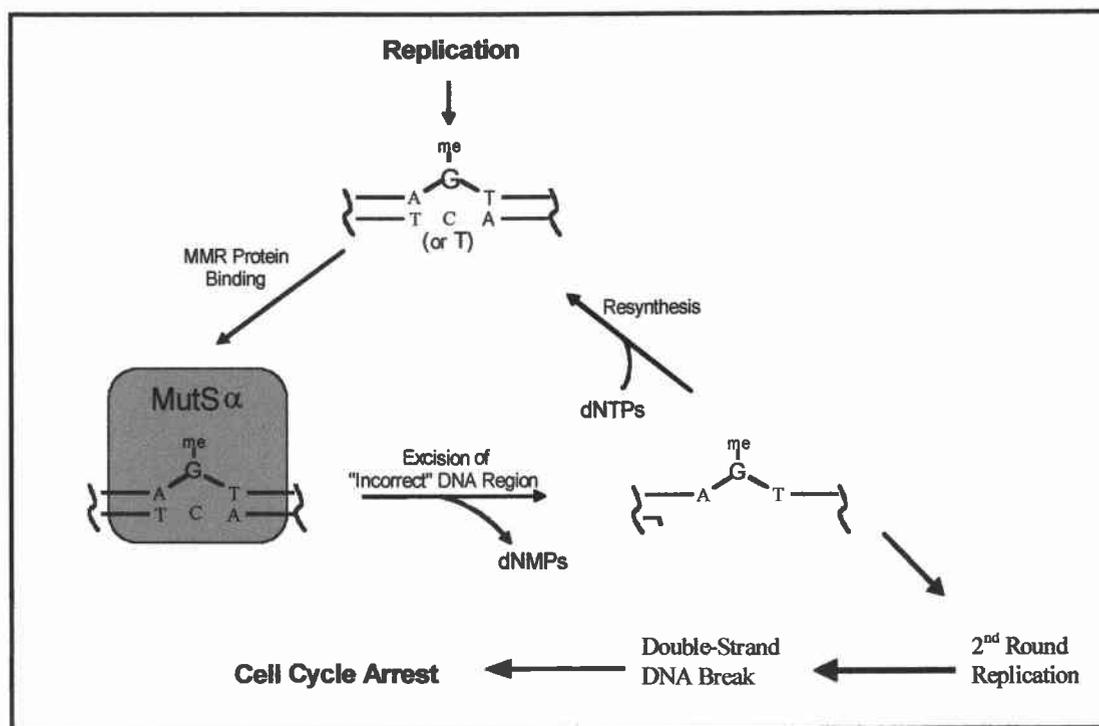


Figure 2: Schematic representation for the Futile DNA Repair Cycle. An O⁶-meG residue present on the template strand of DNA will elicit a repair response by the DNA mismatch repair system regardless of what residue is present on the complementary strand. Following initial excision and repair of the complementary strand, the O⁶-meG will still be present in DNA, triggering a second round of repair. It is proposed that such futile cycling would result in double-strand DNA breaks after a second round of replication, triggering cycle arrest.

Testing the Futile Repair Cycle In Vitro

MMR-proficient cellular extracts are capable of repairing mismatched-DNA substrates containing single specific nicks⁽⁹⁾. The MMR proteins recognize the nicked strand of DNA as if it were the newly synthesized strand *in vivo* and selectively initiate repair of this sequence of DNA. In such a system, the progress of the repair response to O⁶-meG can be monitored by introducing an O⁶-meG-containing plasmid (possessing a nick in its complementary strand) into an MMR-proficient cell-free extract prepared from nuclei of human (HeLa) cells. A specific inhibitor of methyltransferase, O⁶-benzylguanine, would be added to prevent demethylation of the substrate. ³²P-labeled precursors of resynthesis (dCTPs) would be provided, and the generation of labeled excision products (dNMPs) could be measured as evidence of futile cycling (see Figure 2).

One would expect to observe the accumulation of labeled dCMP in the extracts containing the O⁶-MeG substrate, supporting the futile repair cycle model. Such a result would provide mechanistic evidence of the initial signal for apoptotic action by alkylating chemotherapy agents such as TMZ. As previously mentioned, this information would allow for a greater understanding of the processes and dangers involved with the use of such drugs in cancer chemotherapy.

This paper reports preparation progress of the described assay, including a description of the specific techniques involved. Considerations regarding the feasibility of such an assay are also addressed.

Alkaline Phosphatase and Repair Efficiency

A second component of this report addresses the possibility of increasing the efficiency of *in vitro* repair assays. The proposed assay for measuring futile repair cycles is dependent upon high repair efficiencies. One possible way to achieve this is by reducing the effects of the competing cellular process of ligation. Nicked DNA substrate would be expected to be ligated to the closed circular form to some extent when introduced into functional cell extracts. Such ligated substrate will not be processed by MMR, presumably reducing the total amount of repair observed. This is especially relevant for this assay where re-initiation of repair, and therefore retention of the specific nick, is necessary. Removal of the 5'-phosphate from the nicked substrate using alkaline phosphatase would act to block the *in vitro* ligation events. However, there are currently no published studies regarding dephosphorylation of nicked DNA substrate.

In this report, the ability of alkaline phosphatase to block ligation events of nicked substrate is determined following initial assays to establish optimal conditions for this assay. The relative repair efficiency of the dephosphorylated substrate is then compared with that of nicked substrate possessing the 5' phosphate.

Materials and Methods

Escherichia coli Strain: Media and Growth

Except were noted, bacterial work was performed using *E. coli* strain XL1-Blue grown in LB broth (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7) with tetracycline (12.5 µg/ml). LB plates contained 1.5 % agar. Growth of *E. coli* for phage production was performed with the above strain in 2xYT media (1.6% tryptone, 1% yeast extract, 0.5% NaCl, pH 7). When applicable, plasmid selection was accomplished using ampicillin (100 µg/ml). Cell densities were determined by measuring either A₆₀₀ (Beckman DU 640B Spectrophotometer) or Klett units (Klett-Summerson photoelectric calorimeter, model 800-3). All growth was performed at 37°C. Except were noted, all DNA purification was performed using commercial Miniprep/Maxiprep, plasmid preparation kits (Qiagen).

HeLa Cell Line: Media and Growth

HeLa cell line S3 was used in these experiments. Minimum Essential Media (S-MEM, Joklik-modified [GibcoBRL]) supplemented with 5% fetal bovine serum (GibcoBRL), streptomycin (200 µg/ml), and penicillin-G (200U/ml) was used for all cultures. All growth was performed at 37°C. Suspension cultures were first grown in flat culture flasks to approximately 90% confluency (as determined by visual microscopy), trypsinized, and transferred to spinner bottles. Cell densities were determined by Coulter counting (Coulter Electronics, Inc.). Dilution and transfer of suspension cultures were performed at cell densities of approximately 1.0×10^6 cells/ml.

DNA Gel Electrophoresis and Densitometry

All visual DNA analysis was performed with electrophoresis using 1% agarose gel as described⁽¹⁸⁾. Gels contained ethidium bromide (1.25 µg/ml) or, where noted, were post-stained with ethidium bromide solution (0.5 ug/ml). Digital images of gels were captured using an Imagestore 7500, computer-integrated CCD-camera (UVP Inc.). Densitometric analysis of these images was performed using IPLab Gel scientific image processing software (Signal Analytics).

Isolation of Single-Stranded DNA

A phage stock of the M13-derived vector, UVMR1, was obtained from Dr. Huxian Wang (Oregon State University). The UVMR1 vector contains an insert of specific endonuclease restriction sites engineered specifically for preparation of DNA substrates. Isolation of single-stranded DNA (ssUVMR1) was performed according to standard protocols⁽¹⁹⁾. Briefly, 1500 ml of *E. coli* suspension culture was grown to a density of $\sim 5 \times 10^8$ cells/ml and inoculated with UVMR1 phage stock at a ratio of 20 pfu/cell. The culture was incubated for six hours, placed on ice, and centrifuged at for 10 min at 4000g. Supernatant was collected and centrifuged as before to remove cell contaminant. The cell pellet was also collected for preparation of double-stranded UVMR1 (dsUVMR1) vector DNA. Final supernatant (1450 ml) was retained.

NaCl (36 g/L) and PEG-8000 (50 g/L) were added to supernatant and mixed at room temperature for 40 minutes. The phage pellet was collected by centrifuging as before for 30 minutes and allowed to air. The pellet was then resuspended in 5 ml of TE

buffer (10 mM Tris·HCl, pH 8, and 1 mM EDTA) and a phenol/chloroform extraction of DNA was performed. DNA was then dialyzed into TE buffer. Concentration and purity of total DNA was determined by measuring A_{260} and A_{280} (Beckman DU 640B Spectrophotometer). Single-stranded and double-stranded composition was determined by densitometric comparison (post-stained gel) with a low DNA mass ladder (GibcoBRL). A control sample of ssUVMR1 DNA (provided by Dr. Huixian Wang) was also run as a standard.

Thin Layer Chromatography of Adenosine Ribonucleotides

The following samples were applied to a PEI/cellulose thin layer chromatography plate (Sigma): 1) 250 pmol ATP, 2) 250 pmol ADP, 3) 250 pmol AMP, 4) mixture of 250 pmol ATP and 125 pmol AMP, 5) mixture of 250 pmol and 63 pmol AMP, 6) mixture of 250 pmol ATP and 31 pmol AMP. A mobile phase of 1 M formic acid/0.5 M LiCl₂ was used. Samples were visualized with shortwave UV shadowing using a transilluminator (UVP Inc.). Digital images were captured using an Imagestore 7500, computer-integrated CCD-camera (UVP Inc.)

Bacteriophage ϕ 1 Gene II-Protein Purification and Analysis

E. coli strain K1073 (containing the cloned gene II under a *TaqI* promoter) was graciously provided by Dr. Paul Modrich (Duke University Medical Center). Purification of the gene II protein was performed using an abbreviated/modified version of the protocol described by Greenstein, *et al*⁽²⁰⁾. Eliminated in the abbreviated protocol was the second gel filtration step using a Sephacryl 400 column.

Briefly, a 200 ml culture of *E. coli* K1073 in 2xYT medium with ampicillin (100 µg/ml) was grown to $\sim 3.75 \times 10^8$ cell/ml then treated with IPTG (2 mM). After incubation for 7 hours, the culture was centrifuged at 4000 g at 4°C for 15 min and the cell pellet was resuspended in 22.5 ml buffer A (100 mM Maleic acid•NH₄ [pH 6.8], 10% [v/v] glycerol, 1 mM EDTA, 5 mM β-mercaptoethanol). Cells were lysed using a French press and the lysate was centrifuged at 2000 g for 15 min at 4°C (gene II-protein in supernatant). The supernatant from this low-speed spin was then centrifuged for 100,000 g for 60 min at 4°C (gene II-protein in pellet). The pellet was resuspended in 10 ml buffer A with 1 M guanidine HCl and allowed to stir at 4°C for one hour. This mixture was centrifuged for 100,000 g for 60 min at 4°C (gene II-protein in pellet). The pellet was resuspended in 2.5 ml buffer A with 1 M guanidine•HCl then solid guanidine•HCl was added to 7 M (final volume 4 ml). The mixture was allowed to stir at 4°C for one hour. This mixture was centrifuged for 100,000 g for 60 min at 4°C (gene II-protein in supernatant). Two ml of supernatant was loaded onto a Sephacryl 200 filtration column and after eluting 30 ml (theoretical void volume was ~42 ml), sixty 0.5 ml fractions were collected. Every other fraction was analyzed using 12% SDS polyacrylimide gel electrophoresis. Peak fractions were diluted 1:9 in column buffer (7 M guanidine•HCl, 50 mM Tris•HCl [pH 7.4], 1 mM EDTA, 50 mM β-mercaptoethanol). Ten ml of diluted sample was dialyzed into buffer B (25 mM Imidazole•HCl [pH 6.8], 10% [v/v] glycerol, 400 mM KCl, 1 mM EDTA, 5 mM β-mercaptoethanol) with 1 M guanidine•HCl for 2.5 hrs, then buffer B for 24 hrs with four buffer changes. Dialyzed protein samples were aliquoted, flash frozen in liquid nitrogen, and stored at -80°C. Total protein content was determined using commercial Bradford analysis (BioRad, Inc).

Activity assays of the dialyzed gene II-protein were performed using pBSK+ or pUC18 plasmid DNA in modified reaction buffer (20 mM Tris•HCl [pH 8.0], 5 mM CaCl₂, 5 mM DTT, 80 mM KCl). One-hundred ng of DNA was incubated with 33 ng (2 µl) of protein for 30 min at 30°C. The nicked (RFII) or closed-circular (RFI) DNA composition of reaction samples were determined by densitometric analysis. One unit of activity (U) is defined as the amount of enzyme that will convert 250 ng of fl DNA (111 ng pBSK+) from RFI to RFII in 30 min at 30°C⁽²⁰⁾.

HeLa Cell Nuclear Extract

Nuclear extract was prepared as previously described⁽²¹⁾. Ten liters of HeLa 3S cell suspension in log phase (~5.0 x 10⁵ cells/ml) were incubated on ice for 30 min then harvested by centrifugation at 3000 g for 15 min. Cell pellet was resuspended in 200 ml of wash buffer (20 mM HEPES•KOH [pH 7.5], 5 mM KCl, 0.5 mM MgCl₂, 0.2 mM sucrose, 0.1% PMSF, 0.5 mM DTT, 1 µg/ml leupeptin) and centrifuged at 3300 g for 5 min. Cell pellet (~9.5 gm) was resuspended in 27 ml of hypotonic buffer (20 mM HEPES•KOH [pH 7.5], 5 mM KCl, 0.5 mM MgCl₂, 0.1% PMSF, 0.5 mM DTT, 1 µg/ml leupeptin) and incubated on ice for 10 min. Cell viability was verified by staining cells with trypan blue and observing under a microscope, then cells were lysed by douncing cells on ice for ten strokes. Intact nuclei were verified by observing under a microscope, then pelleted by centrifuging at 2000 g for 5 min.

Nuclei were resuspended in 13ml cold extraction buffer (50 mM HEPES•KOH [pH 7.5], 10% sucrose, 0.1% PMSF, 0.5 mM DTT, 1µg/ml leupeptin) and NaCl was added to a final concentration of 0.155 M. Suspension was rotated at 4°C for one hour then

centrifuged at 14,500 g for 20 min. The supernatant (11 ml) was collected and 4.6 g of ammonium sulfate slowly added over 20 min with stirring on ice, then allowed to stir for an additional 20 min. The nuclear proteins were then pelleted by centrifuging at 15,800 g for 20 min. The protein pellet was transferred directly to dialysis tubing (Spectra/Por #2, Spectrum Medical Industries, Inc.) and dialyzed against TE buffer to a final conductivity of ~40 $\mu\text{S}/\text{cm}$ (CDM 80 conductivity meter, Radiometer/Copenhagen). Dialyzed protein extract was aliquoted, flash frozen in liquid nitrogen, and stored at -80°C . Total protein content was determined using commercial Bradford analysis (BioRad, Inc).

In Vitro Repair Assays

Repair assays were performed using G/T mispaired heteroduplex substrate, graciously provided by Dr. Huixian Wang (Oregon State University), as described⁽⁹⁾. One-hundred ng of heteroduplex DNA was added to 50 μg of total extract protein in reaction buffer (20 mM Tris \cdot HCl [pH 7.6], 50 $\mu\text{g}/\text{ml}$ BSA, 1.5 mM ATP, 1 mM reduced glutathione, 0.1 mM dNTPs, 5 mM MgCl_2). Reactions were mixed, centrifuged briefly, and incubated at 37°C for 15 min. Ten μl reactions were stopped by addition of 30 μl proteinase K stock (0.1 mg/ml proteinase K, 25 μM EDTA, 0.7% SDS) and incubating at 37°C . DNA was then extracted by phenol/chloroform extraction twice, ether extraction twice, then ethanol precipitation.

DNA samples were then treated in a double-restriction digestion mixture (2U *Hind*III [restriction] endonuclease, 2U *Cla*I [restriction] endonuclease, 20 mM Tris \cdot HCl [pH 8.0], 50 mM KCl, 2mM DTT, 10 $\mu\text{g}/\text{ml}$ BSA, 10 mM MgCl_2) at 37°C for 60 minutes. Densitometric analysis (post-stained gel) was then performed. Control

standards used were dsUVMR1 DNA for restriction control and *Cla*I-treated heteroduplex DNA for the linear control.

BAP-C and Ligation Assays

Bacterial alkaline phosphatase (BAP-C, Fermentas) reactions using synthetic substrate (p-nitrophenyl phosphate or pNPP) were performed with 0.001 unit (per 200 μ l sample) and 1.5 mM pNPP in 50 mM Tris•HCl (pH 9.0). The standard $MgCl_2$ concentration was 1 mM. For $MgCl_2$ -dependence assays, concentrations were varied from 0.01 mM to 1 mM. 37°C and 80°C incubation temperatures were obtained using a water bath or thermal cycler (Biometra personal cycler), respectively.

DNA substrate for nicking assays (pUC18NE) was provided by Dr. Huxian Wang (Oregon State University). This substrate possesses a single recognition site for the *N.Bst*NI endonuclease. Two-hundred μ g of this DNA was incubated for one hour at 55°C with 5 units of *N.Bst*NI endonuclease (New England Biolabs) in NEBuffer 2 (10 mM Tris•HCl [pH 7.9], 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT). This sample was then incubated at 80°C for 20 minutes to inactivate enzyme.

BAP-C treatment of DNA (for both the ligation studies and repair efficiency assays) was performed using 0.1 unit of BAP-C per 100 ng DNA. This was added directly to the *N.Bst*NI reaction samples or to heteroduplex DNA samples in assay buffer (50 mM Tris•HCl, 10 mM $MgCl_2$). Samples were incubated at 70°C for 60 minutes and DNA was recovered by phenol/chloroform extraction and ethanol precipitation.

Each ligation reaction with nicked DNA substrate consisted of 200 ng DNA with 2.5 units of T4 DNA ligase (GibcoBRL) in reaction buffer (50 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 25 µg/ml BSA). The nicked (RFII) or closed-circular (RFI) DNA composition of reaction samples was determined by densitometric analysis.

Experimental Results and Discussion

Preparation of O⁶-Methylguanine DNA substrate: An Overview

The general procedure for generating the DNA substrate to be used in this experiment is outlined in Figure 3. An O⁶-MeG-containing oligonucleotide is annealed to single-strand phage DNA template. An extension/ligation reaction is performed followed by CsCl/EtBr banding purification to isolate closed circular (RFI) DNA. A nick is then introduced in the template strand 284 bp 3' to the O⁶-MeG residue using fl gene II product. This nick is dephosphorylated by treatment with alkaline phosphatase.

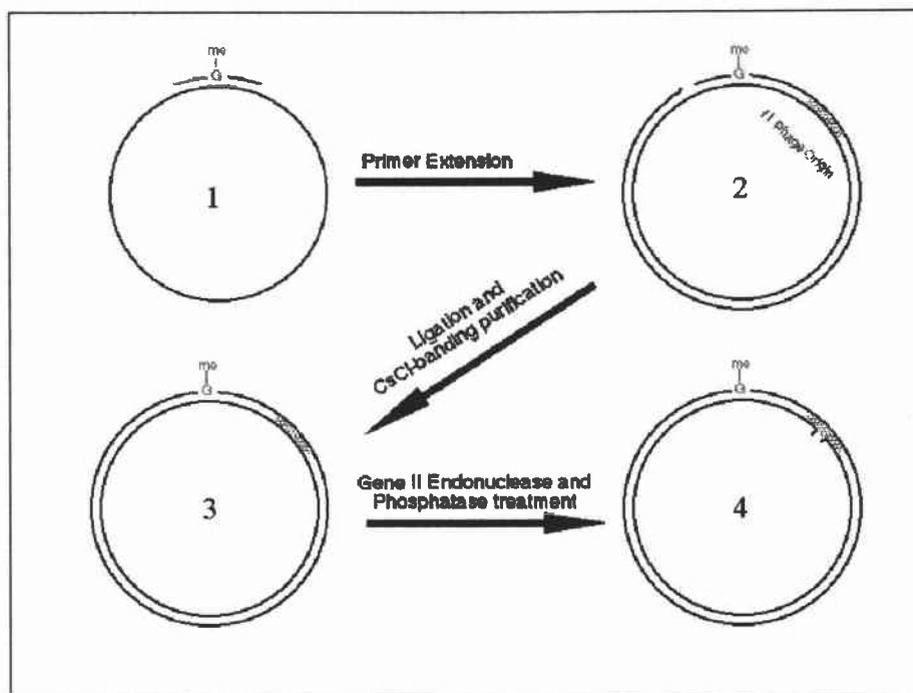


Figure 3: Scheme for the Preparation of O⁶-meG-Containing DNA Substrate: 1) An O⁶-meG-containing oligonucleotide is annealed to a single-stranded phage DNA template, 2) A primer extension reaction is performed yielding the incomplete heteroduplex DNA, 3) Following ligation a CsCl/EtBr-banding purification is performed to yield closed-circular (RFI) heteroduplex DNA, 4) Finally, the substrate is treated with fl phage gene II product followed by alkaline phosphatase to yield nicked (RFII) heteroduplex DNA substrate, lacking a 5' phosphate.

Sufficiently Pure Single-Stranded Template DNA was Prepared

Approximately 2.5 mg of single-stranded UVMR1, phage DNA was isolated with an $A_{260}:A_{280}$ ratio of 1.81, indicating there was no significant protein contamination. This DNA was analyzed for double-stranded DNA contamination using agarose gel electrophoresis and densitometric analysis standardized with a commercial DNA mass ladder (Gibco-BRL). An average of approximately 20 ng of contaminating double-stranded DNA was detected per 500 ng total DNA of combined preparations (as determined by comparison with the 40 ng band of the DNA mass ladder--Figure 4). This corresponds to approximately 4% contaminant, a level acceptable for use in extension/ligation protocols⁽²²⁾.

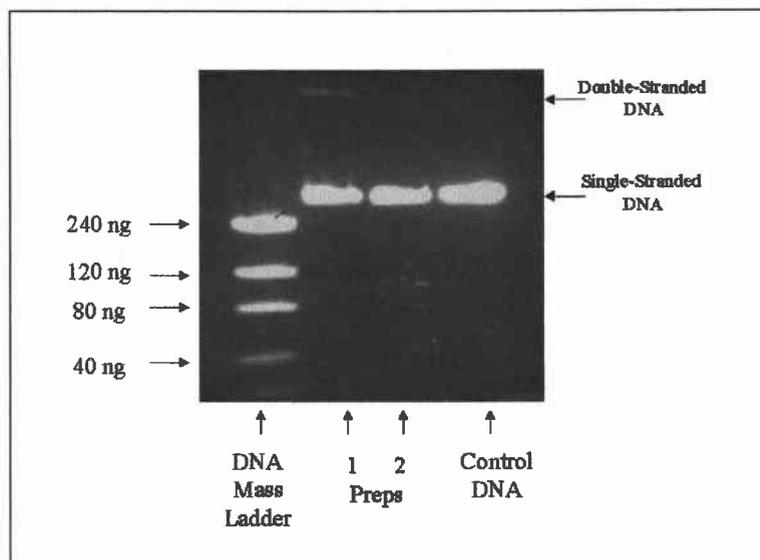


Figure 4: Purity Analysis of Single-Stranded Phage DNA. A total quantity of 500 ng of purified single-stranded phage DNA (ssUVMR1) from two separate preparations (lanes 2 and 3) were run on a 1.25% agarose gel. A double-stranded DNA mass ladder (lane 1) and a previously purified control sample of ssUVMR1 (lane 4) were also analyzed. Densitometric comparison with the 40 ng band of the mass ladder was used to determine the total amount of contaminating double stranded DNA present in each preparation. Each preparation was determined to have less than 30 ng of double stranded contamination (approximately 4 % total contaminant when combined).

f1 Gene II Product was Purified with a Specific Activity of 1.1×10^4 U/mg

The gene II product of f1 phage is a site specific, single-strand endonuclease that nicks specifically at f1 phage origins⁽²⁰⁾. As described, an abbreviated version of the published purification protocol for this enzyme was used in this experiment. Excluded in our protocol was a second gel filtration step, raising the concern of possible non-specific, contaminating activity. The elution profile from the single filtration column is shown in Figure 5. The specificity of the final, dialyzed peak fractions was determined by incubating pBSK+ plasmid (possesses a f1 origin) or pUC18 (no f1 origin) in reaction buffer with 2 μ l of enzyme sample. These samples were analyzed using agarose gel electrophoresis with EtBr-saturation, allowing for separation of nicked (RFII) from closed-circular (RFI) DNA. As shown in Figure 6, activity was specific for the f1 origin containing DNA substrate (pBSK+).

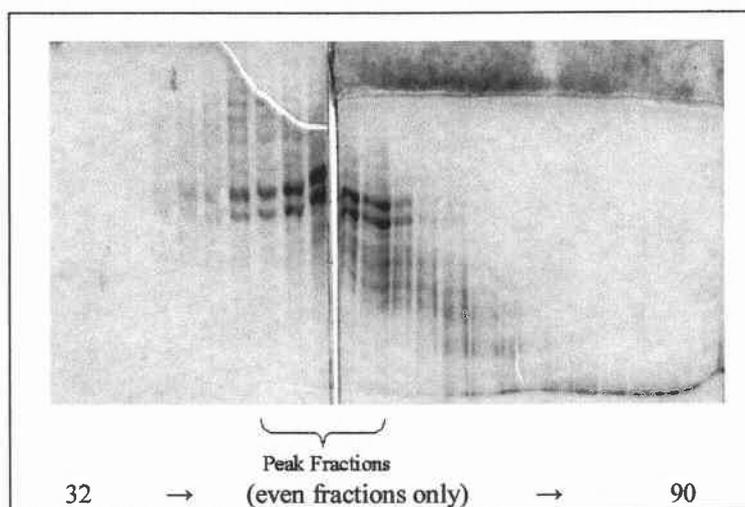


Figure 5: SDS/PAGE Elution Profile of Gene II Gel Filtration. Following initial separation steps, the denatured protein preparation was loaded onto a Sephacryl 200 column. After the void volume (~30 ml, 0.5 ml fractions were collected and every other fraction was analyzed using SDS/PAGE. The two prominent bands halfway down migrate at the proper molecular weight (~40kD, size standard not shown) and are believed to be the gene II protein. Highlighted are the peak fractions chosen for dialysis.

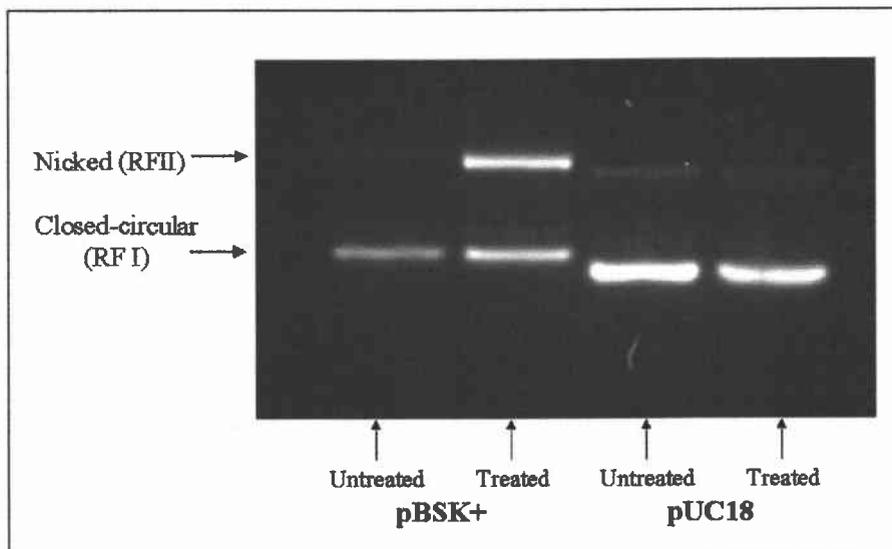


Figure 6: Substrate Specificity of Gene II Protein Preparation. Reactions using the dialyzed peak fractions (see Figure 5) were performed with pBSK+ (possesses fl phage origin) and pUC18 (no fl phage origin). Nicking of pUC18 was not observed.

To determine the specific activity of the gene II-protein sample, 100 ng of pBSK+ was incubated with 33 ng (2 μ l) of dialyzed sample for 5, 10, 15, and 30 minutes (Figure 7). Although activity dramatically decreased with time, the overall specific activity for a 30-minute incubation was roughly 1.1×10^4 U/mg of protein. This value is less than 5% of the published specific activity reported for the complete protocol⁽²⁰⁾. This lower ratio of enzymatic activity to protein concentration is most likely due to the elimination of the second chromatography step resulting in a greater amount of total protein in this sample (therefore, a smaller proportion of the measured protein mass is the desired enzyme).

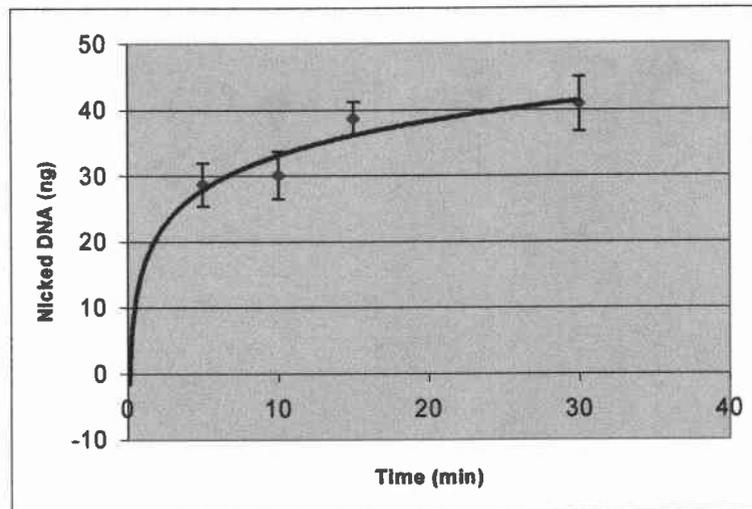


Figure 7: Kinetic Analysis of ϕ Phage Gene II Product. One-hundred ng of pBSK+ plasmid DNA was treated with 33 ng (2 μ l) of purified gene II sample. Reactions were allowed to proceed for 0, 5, 10, 15, and 30 minutes. Activity was quantified by densitometric determination of the amount of RFII (nicked) DNA produced from RFI; 1U equals 250 ng of phage (or 111 ng pBSK+) DNA converted from RFI to RFII in 30 min at 30°C. The overall specific activity for this protein preparation corresponded to 1.1×10^4 U/mg.

Ribonucleotides are Efficiently Separated by Thin Layer Chromatography

To determine whether a mixture of radiolabeled dNTPs and dNMPs could be separated by thin layer chromatography methods (a necessary procedure for observing futile repair cycles as described), an assay was performed using ATP, ADP, AMP, and three mixtures of ATP/AMP (Note: Ribonucleotides were used in this assay instead of deoxyribonucleotides because of commercial availability. Because the primary factor influencing chromatographic migration is charge, differences between these compounds should be negligible). These samples were analyzed by PEI/cellulose thin layer chromatography plates and visualized by ultraviolet shadowing (Figure 8). All three nucleotides migrated at very distinct locations. ATP and AMP show the greatest separation (with AMP migrating near the solvent front and ATP barely moving from the

initial position) and are easily distinguished in all mixtures of the two. Since this separation appears to be primarily a function of phosphate charge, deoxyribonucleotides are expected to behave identically, making this assay applicable for use in the described futile cycling assay.

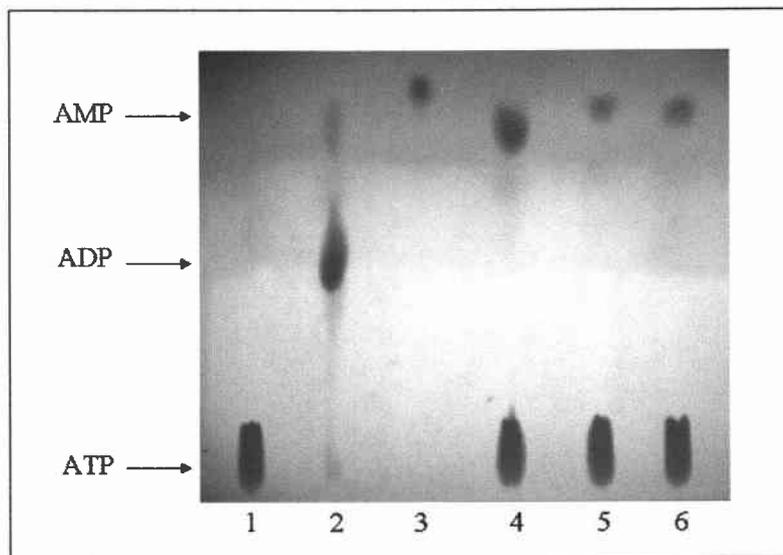


Figure 8: Separation of Ribonucleotides by Thin Layer Chromatography. Adenosine ribonucleotides were run on a PEI/cellulose chromatography plate as follows: 1) 250 pmol ATP, 2) 250 pmol ADP, 3) 250 pmol AMP, 4) 250 pmol ATP and 125 pmol AMP (mix), 5) 250 pmol ATP and 63 pmol AMP (mix), 6) 250 pmol ATP and 31 pmol AMP (mix).

HeLa Cell Nuclear Extracts were Prepared with 30% Repair Efficiency

Initial repair assays of the purified nuclear extract were performed using G/T mismatched, heteroduplex DNA substrate. The mismatch in this DNA substrate is located in a unique *Hind*III recognition site such that correction of the mismatch results in sensitivity to *Hind*III endonuclease, while unrepaired DNA remains resistant to cleavage. In these assays, 100 ng of heteroduplex DNA was incubated in various quantities of nuclear extract (0, 50, or 100 μ g total protein). Following incubation in the extract, the

DNA substrate was extracted and treated with a double-digestion mixture containing *Clal* endonuclease, which linearizes the substrate, and *HindIII* endonuclease, which cleaves repaired DNA into two distinct fragments.

Figure 9 shows the results of the initial assays, including control samples. While two distinct cleavage bands could not be distinguished in the experimental repair samples, a very diffuse band was observed which corresponded to the migration position of the cleaved bands seen for the positive control (lane 3 of Figure 9). This band was assumed to be the repaired population. Densitometric analysis yielded a calculated repair efficiency of approximately 30% for the 50 μg sample. While this measured efficiency is not optimal, it is in the range reported for previously published repair extracts⁽²¹⁾.

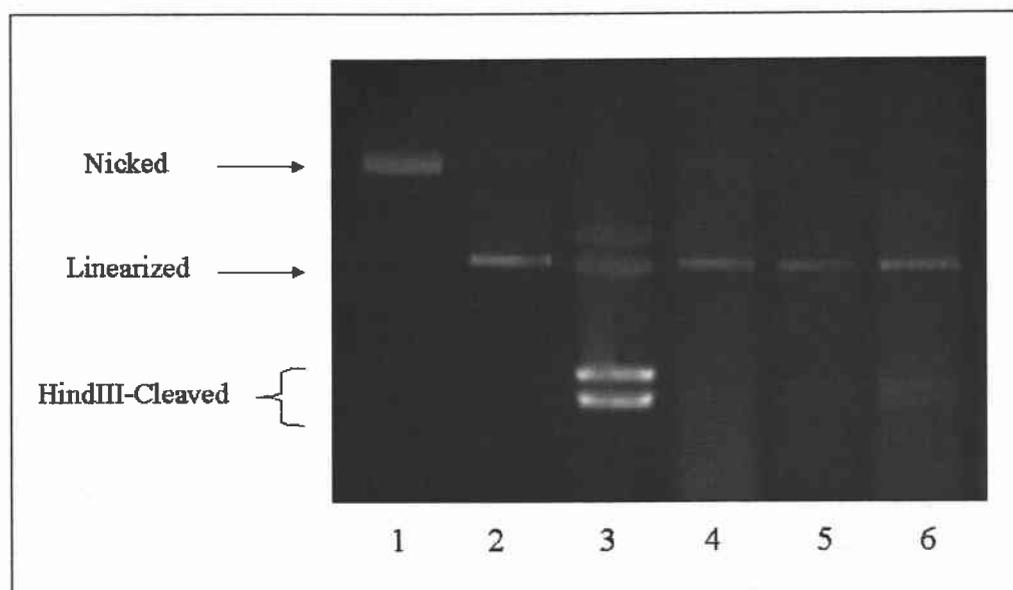


Figure 9: Initial Repair Assays of HeLa Cell Nuclear Extract. Repair reactions and subsequent digestion assays were performed on 100 ng of G/T mispaired heteroduplex DNA using 0, 50, and 100 μg of HeLa cell nuclear extract (lanes 4, 5, and 6, respectively). Control samples are as follows: 1) Nicked heteroduplex DNA (untreated); 2) Digested, unrepaired heteroduplex DNA; 3) Digested homoduplex control DNA. The diffuse bands visible in lanes 5 and 6 (migrating near the *HindIII*-cleaved bands of lane 3) are believed to be the repaired DNA from these samples.

Alkaline Phosphatase Efficiently Blocks Ligation of Nicked DNA Substrate

As discussed earlier, this author is aware of no published studies regarding dephosphorylation of nicked DNA substrates. A primary concern regarding this process is that the alkaline phosphatase enzyme might not have access to the 5' end of the nicked strand. It was proposed then, that reaction conditions would need to be such that the nicked ends of the DNA would separate from the double-stranded substrate and freely protrude into solution. In other words, conditions that reduce DNA annealing would be necessary. Such conditions include high temperature, high pH, and low salt concentrations⁽²³⁾.

To address the temperature factor, bacterial alkaline phosphatase (BAP-C) was selected in place of the commonly used calf intestinal alkaline phosphatase (CIAP) due to the thermostability of BAP-C. While CIAP is deactivated at temperatures greater than 70°C, BAP-C is stable to temperatures of 80°C⁽²⁴⁾. BAP-C is also active in buffers up to pH 9. Assays using the synthetic, phosphatase substrate, p-nitrophenol phosphate (pNPP), were performed to compare enzyme activity. Upon cleavage of its phosphate group, this compound becomes yellow in color. The measured A_{410} of reaction mixtures is thus proportional to the molar quantity of cleaved phosphate groups.

Parallel reactions were performed at 80°C and 37°C, with reaction times of 0, 15, 30, and 60 minutes (Figure 10). The reaction rate observed at 80°C was approximately 5-fold higher than at 37°C. A marked decrease in reaction rate with time was however noted for the 80°C samples. To determine whether this decrease was due to inactivation of the enzyme or exhaustion of pNPP substrate, a second series of 80°C reactions was performed using a 10-fold reduction in enzyme concentration (Figure 11). Reactions

under these conditions remain essentially linear with time, suggesting that exhaustion of substrate was responsible for the initial decrease in reaction rate observed.

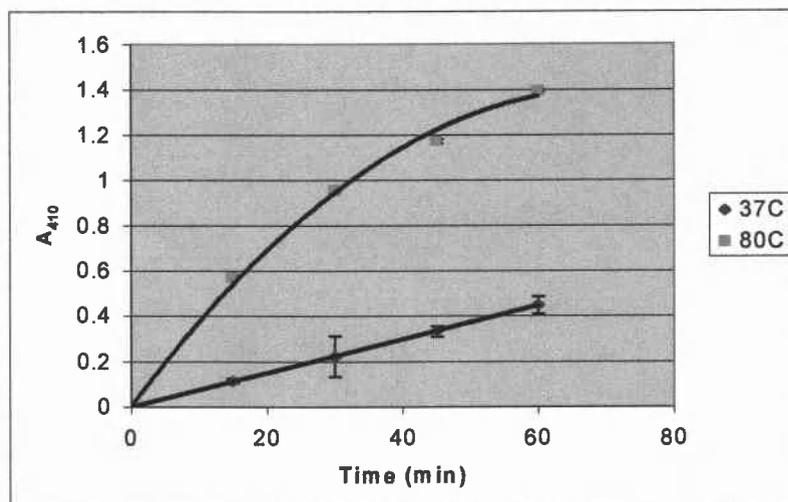


Figure 10: BAP-C Temperature Dependence. Parallel BAP-C assays were performed at 37°C and 80°C using pNPP synthetic substrate. Reactions were stopped after 0, 15, 30, 45, and 60 minutes, and A_{410} readings were taken. Comparison of initial reaction rates shows approximately a 5-fold increase in activity at 80°C.

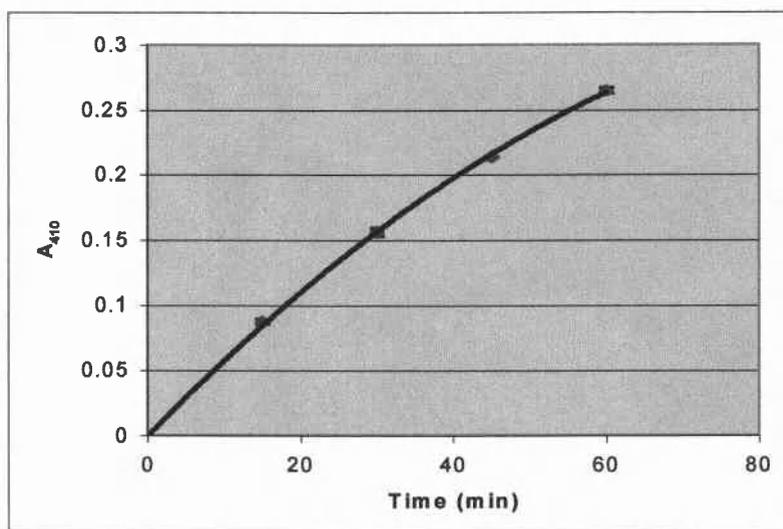


Figure 11: Secondary BAP-C Assay at 80°C. BAP-C reactions were repeated at 80°C using a 10-fold reduction in enzyme quantity. The activity curve obtained is much more linear than the initial activity measurements, suggesting that substrate exhaustion, and not enzyme deactivation, was responsible for the original depression in reaction rate with time.

With the exception of buffer salts, the only salt contribution in the phosphatase reaction mixtures is the MgCl_2 enzyme cofactor, with a standard concentration of 1 mM. Assays were therefore performed at 37°C using MgCl_2 concentrations of 1 mM, 0.1 mM, 0.01 mM to assess the effects of decreased salt concentration on enzyme activity (Figure 12). The reaction rates observed at 0.1 and 0.01 mM show no significant difference from one another and are identical to rates observed in previous reaction assays using 1mM MgCl_2 . Therefore, it is concluded that BAP-C assays can be performed using MgCl_2 concentrations as low as 0.01 mM with no significant loss of activity. It should also be noted that DNA plasmid preparations were exposed to the optimized BAP-C nicking conditions (80°C and 0.01 mM MgCl_2) for up to one hour with no non-specific nicking of DNA noted (data not shown).

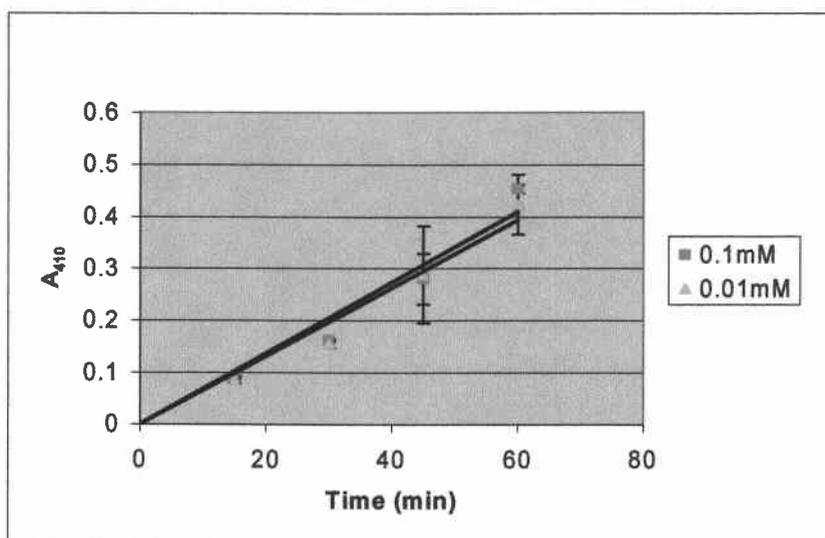


Figure 12: BAP-C MgCl_2 Dependence. BAP-C assays were performed at 37°C using 0.1 mM and 0.01 mM concentrations of MgCl_2 . Reactions were stopped at 0, 15, 30, 45, and 60 minutes. The reaction rates observed are identical to that of previous assays using 1 mM MgCl_2 .

Finally, the ability of BAP-C to block ligation events was assessed. Single specific nicks were introduced into the DNA substrate by treatment with the commercial enzyme N.BstNBI. Half of this DNA was treated with BAP-C at 70°C, then purified via phenol/chloroform extraction and ethanol precipitation. DNA samples were then treated with T4 DNA ligase. Analysis using EtBr-saturated, agarose gel electrophoresis and densitometry was performed to determine relative nicked (RFII) and closed circular (RFI) populations (Figure 13). Approximately 90% of the nicked DNA not treated with BAP-C shifting from RFII to RFI, indicating efficient ligation of nicked strands. BAP-C treated DNA, in contrast, showed no detectable shift. Therefore, treatment with BAP-C appears to efficiently block ligation events in nicked DNA.

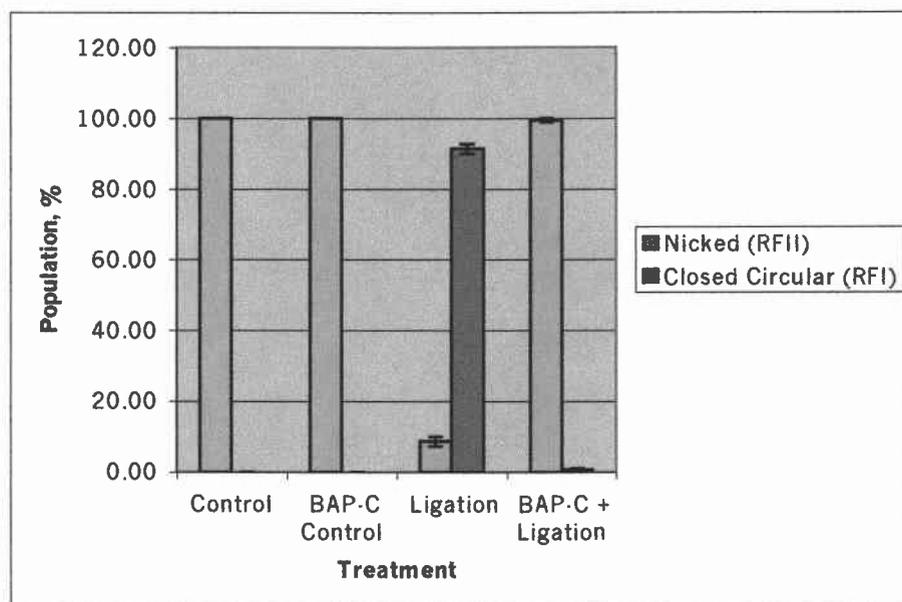


Figure 13: Blocking of Ligation Events by Treatment with BAP-C. Nicked (RFII) DNA was treated with BAP-C at 80°C. Both BAP-C treated and untreated DNA samples were then subjected to *in vitro* ligation reactions. Agarose gel electrophoresis and densitometric analysis was used to determine relative amounts of RFI and RFII DNA in each sample. While a large shift to RFI is seen for the untreated, ligation samples, BAP-C treated DNA shows no detectable shift.

Alkaline Phosphatase Treatment Does Not Appear to Increase In Vitro Repair Efficiencies

Nicked, G/T heteroduplex DNA was treated with BAP-C as above. Following extraction of the DNA, *in vitro* repair reactions were performed on both BAP-C treated and untreated DNA as before (using 50 μ g nuclear extract). Agarose gel electrophoresis and densitometric analyses yielded measured repair efficiencies of approximately 40% (\pm 3.6%) and 33% (\pm 3%) for BAP-C treated and untreated samples, respectively (Figure 14). It should be noted that this difference does not appear to be significant. Furthermore, it was discovered that DNA amounts in repair reactions were not identical for the treated and untreated samples. This discrepancy was the result of a failure to normalize DNA amounts following BAP-C treatment, resulting in less BAP-C treated DNA (~20% less) being used in the subsequent repair reactions.

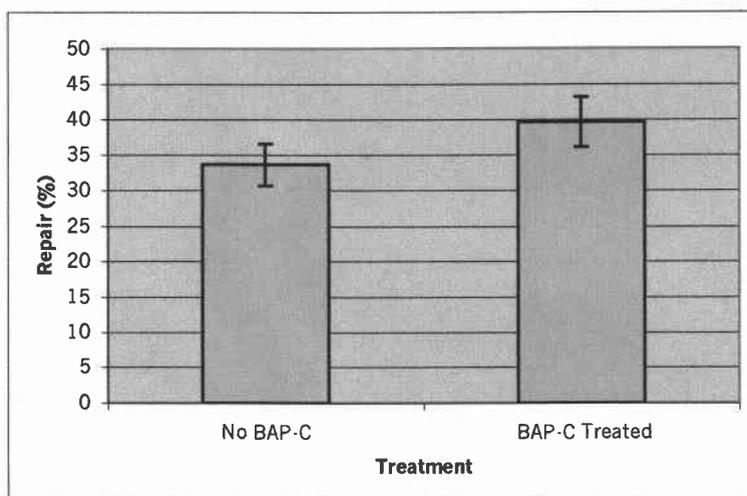


Figure 14: The Effects of BAP-C Treatment on *in Vitro* Repair. Nicked G/T heteroduplex DNA was treated with BAP-C at 80°C for 30 minutes. Following phenol/chloroform extraction and ethanaol precipitation, this DNA was used for *in vitro* repair reactions. Repair assays were also performed on non-BAP-C treated DNA. Restriction digestion, gel electrophoresis, and densitometric analysis was then performed to determine the relative repair efficiencies. Although a slight increase in repair efficiency was observed for BAP-C treated DNA, this is most likely the result of a difference in the relative amount of DNA used in these repair reactions (see text).

In conclusion, while an increase in the measured repair efficiency was noted for the BAP-C treated DNA, this apparent increase was probably due to a smaller quantity of this DNA being added to the repair assays relative to the total amount of protein in these reactions. If an actual increase of repair efficiency did occur during these assays, it was presumably small and probably not sufficient to greatly affect the overall repair signal. However, these results can only be considered valid for single-round repair processes. It is still not understood how dephosphorylation would effect multiple-round repair processes such as futile repair cycling.

Further Study is Necessary to Determine the Mechanism of MMR-Dependent Resistance to DNA Alkalating Agents

The final steps of DNA substrate preparations are still necessary for testing of the futile repair cycle hypothesis. Specifically the extension/ligation and CsCl/EtBr banding purification steps are required. Optimization of the extension/ligation reactions has been performed (data not shown), but efficient recovery of closed circular (RFI) substrate following CsCl/EtBr banding has not yet been achieved. It is not clear why this process has not succeeded and further work is required. Also, as previously discussed, efficient nicking of DNA substrate using the purified preparation of gene II product is questionable due to the low level of activity obtained. Experiments are currently being conducted to achieve higher activity preparations.

Questions regarding the assay itself can also be raised. Primarily, it is possible that even if futile cycling occurs *in vitro*, the signal of such a process may not be

measurable above the background effects of dNTPases expected to be present in the cell extracts. Rough calculations yield a turnover rate of approximately 1.2 pmol of radiolabeled dCMP per repair cycle under standard *in vitro* repair conditions (based on 100% repair efficiencies). Actual values would be expected to be 0.36 pmol dCMP per cycle, based on 30% repair efficiency. This also assumes that previously measured repair efficiencies are maintained for successive rounds of replication. While these quantities of dCMP are within the sensitivity of ^{32}P detection assays, it is very possible that background degradation of α - ^{32}P -dCTP to α - ^{32}P -dCMP in cell extract would drown such a signal. This may not in fact be a concern however, because the primary degradation product in cellular extract would be dCDP which, as shown in Figure 9 can be easily distinguished from dCMP. Further calibration experiments will be necessary to determine background degradation effects.

The mechanism of MMR-dependent resistance to the apoptotic effects of DNA alkylating agents is still not understood. The assay described in this report is perhaps the only method for directly testing the most popular hypothesis for this phenomenon, the futile repair cycle model. This assay should therefore continue to be pursued regardless of possible technical difficulties.

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