1995

Analysis of Amino Acids Relevant to Activity and Substrate Specificity of the Human DNA Repair Protein 0-6-Methylguanine DNA Methyltransferase (MGMT)

Susan Elizabeth Morgan
Loyola University Chicago

Follow this and additional works at: https://ecommons.luc.edu/luc_diss

Part of the Molecular Biology Commons

Recommended Citation
https://ecommons.luc.edu/luc_diss/3383

This Dissertation is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Dissertations by an authorized administrator of Loyola eCommons. For more information, please contact ecommons@luc.edu.

This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License.
Copyright © 1995 Susan Elizabeth Morgan
ANALYSIS OF AMINO ACIDS RELEVANT TO ACTIVITY AND SUBSTRATE SPECIFICITY OF THE HUMAN DNA REPAIR PROTEIN 0-6-METHYLGUIANINE DNA METHYLTRANSFERASE (MGMT)

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

GRADUATE PROGRAM IN MOLECULAR BIOLOGY

BY

SUSAN ELIZABETH MORGAN

CHICAGO, ILLINOIS

JANUARY, 1995
Copyright by Susan E. Morgan, 1994
All rights reserved
ACKNOWLEDGEMENTS

I would like to recognize the members of my committee for their helpful criticisms and assistance in developing a productive graduate school career: Dr. M. Eileen Dolan, Dr. Leonard C. Erickson, Dr. Mark R. Kelley, Dr. John M. Lopes, and my advisor Dr. Russell O. Pieper. I would also like to thank Russ for his constant guidance, support, and dedication. I am grateful for the influence Russ has had on my scientific presentations, writing, and critical thinking.

To my friends and laboratory colleagues, I also offer my appreciation for their help and camaraderie: Dr. Joseph Costello, Dawn Graunke, Tanja Dubrovic, Sonal Patel, and Janice Kerr.

I would like to acknowledge all those who assisted me in the preparation of this dissertation. In particular, I thank Fran Jannece and Dee Miller for their patience and aid throughout my stay here at Loyola.

Finally, I would like to extend special thanks to Christopher Lappe and to my parents, John and Carole Morgan.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xi</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xiv</td>
</tr>
<tr>
<td>Chapter</td>
<td></td>
</tr>
<tr>
<td>I.  INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. REVIEW OF THE RELATED LITERATURE</td>
<td>6</td>
</tr>
<tr>
<td>A. DNA Alkylating Agents</td>
<td>6</td>
</tr>
<tr>
<td>B. O₆-Methylguanine DNA Methyltransferase (MGMT)</td>
<td>10</td>
</tr>
<tr>
<td>C. Escherichia coli Alkyltransferase (Ada)</td>
<td>12</td>
</tr>
<tr>
<td>D. MGMT and Ada Protein Structure and Function</td>
<td>14</td>
</tr>
<tr>
<td>E. MGMT and Ada Substrate Specificity</td>
<td>19</td>
</tr>
<tr>
<td>1. Differential repair of various sized O₆-alkylguanine adducts by MGMT and Ada</td>
<td>20</td>
</tr>
<tr>
<td>2. Differential repair of O₄-methylthymine and O₆-benzylguanine by MGMT and Ada</td>
<td>21</td>
</tr>
<tr>
<td>III. MATERIALS AND METHODS</td>
<td>25</td>
</tr>
<tr>
<td>A. Bacteria and Plasmids</td>
<td>25</td>
</tr>
<tr>
<td>B. Cell Culture</td>
<td>25</td>
</tr>
<tr>
<td>C. Generation of Full-Length, Truncated, and Chimeric MGMT and ada cDNAs by PCR Amplification</td>
<td>26</td>
</tr>
<tr>
<td>Chapter</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
</tr>
<tr>
<td>D. Preparation of Competent Alkyltransferase Deficient <em>E. coli</em> Cells</td>
<td>33</td>
</tr>
<tr>
<td>E. Subcloning of MGMT and <em>ada</em> cDNAs into a Bacterial Expression Vector</td>
<td>33</td>
</tr>
<tr>
<td>F. Isolation of GST-MGMT, GST-Ada, and GST-Chimeric Fusion Proteins from <em>E. coli</em></td>
<td>35</td>
</tr>
<tr>
<td>G. Staining of SDS-Polyacrylamide Gels</td>
<td>37</td>
</tr>
<tr>
<td>H. Protein Quantitation</td>
<td>38</td>
</tr>
<tr>
<td>I. Preparation and Labeling of an 18 bp O$\alpha$-MG-Containing DNA Substrate</td>
<td>39</td>
</tr>
<tr>
<td>J. Assay for Repair of O$\alpha$-MG in DNA</td>
<td>42</td>
</tr>
<tr>
<td>K. Analysis of O$\alpha$-BG Repair Activity</td>
<td>43</td>
</tr>
<tr>
<td>L. Assay for Repair of O$\alpha$-MT in DNA</td>
<td>45</td>
</tr>
<tr>
<td>IV. RESULTS</td>
<td>48</td>
</tr>
<tr>
<td>A. Construction of Full-Length, Truncated, and Chimeric MGMT and Ada Fusion Proteins</td>
<td>48</td>
</tr>
<tr>
<td>1. Construction of GST-MGMT, GST-Ada, and GST-chimeric bacterial expression vectors</td>
<td>48</td>
</tr>
<tr>
<td>2. Expression and isolation of GST-MGMT, GST-Ada, and GST-chimeric fusion proteins</td>
<td>50</td>
</tr>
<tr>
<td>B. The Role of the 28 Amino Acid Carboxyl-Terminal Tail in MGMT Activity, Temperature Sensitivity, and Substrate Specificity</td>
<td>60</td>
</tr>
<tr>
<td>1. Repair of O$\alpha$-MG in DNA by control MGMT and carboxyl-terminal MGMT deletion proteins</td>
<td>60</td>
</tr>
<tr>
<td>2. Quantitation of active MGMT and MGMT-C28 fusion proteins</td>
<td>65</td>
</tr>
<tr>
<td>Chapter</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
</tr>
<tr>
<td>3. Effect of 28 amino acid carboxyl-terminal tail deletion on the rate of MGMT-mediated repair of ( O^6)-MG-containing DNA at 4°C</td>
<td>67</td>
</tr>
<tr>
<td>4. Effect of 28 amino acid carboxyl-terminal tail deletion on the rate of MGMT-mediated repair of ( O^6)-MG-containing DNA at 37°C</td>
<td>69</td>
</tr>
<tr>
<td>5. Effect of 28 amino acid carboxyl-terminal tail deletion on the rate of MGMT-mediated repair of ( O^6)-BG</td>
<td>72</td>
</tr>
<tr>
<td>6. Repair of ( O^6)-BG by ADA-MGMTTAIL fusion protein</td>
<td>74</td>
</tr>
<tr>
<td>C. Analysis of MGMT Amino Acids Required for the Repair of ( O^6)-MG in DNA</td>
<td>75</td>
</tr>
<tr>
<td>D. Analysis of MGMT Amino Acid Regions Involved in ( O^6)-Benzylguanine Substrate Specificity</td>
<td>81</td>
</tr>
<tr>
<td>1. Repair of ( O^6)-MG in DNA by control MGMT, Ada, or MGMT-ADA and ADA-MGMT chimeric fusion proteins</td>
<td>81</td>
</tr>
<tr>
<td>2. Quantitation of active control and chimeric Ada and MGMT fusion proteins</td>
<td>85</td>
</tr>
<tr>
<td>3. Repair of ( O^6)-BG by MGMT-ADA and ADA-MGMT chimeric fusion proteins</td>
<td>89</td>
</tr>
<tr>
<td>4. Repair of ( O^6)-BG by MGMT-ADA-MGMT chimeric fusion proteins</td>
<td>91</td>
</tr>
<tr>
<td>E. Comparison of Ada Amino Acid Regions Involved in ( O^4)-Methylthymine Substrate Specificity to that of MGMT Amino Acid Regions Involved in ( O^6)-Benzylguanine Substrate Specificity</td>
<td>96</td>
</tr>
<tr>
<td>V. DISCUSSION</td>
<td>102</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>117</td>
</tr>
<tr>
<td>VITA</td>
<td>131</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Alkyltransferase sequence alignment</td>
<td>15</td>
</tr>
<tr>
<td>2. Alkyltransferase sequence alignment</td>
<td>24</td>
</tr>
<tr>
<td>3. Specific sequence of the oligodeoxynucleotide probe</td>
<td>41</td>
</tr>
<tr>
<td>4. PCR amplification of control and mutant MGMT and <em>ada</em> cDNAs</td>
<td>49</td>
</tr>
<tr>
<td>5. Diagrams of the full-length, truncated, and chimeric MGMT and Ada fusion proteins and MGMT oligopeptides</td>
<td>51</td>
</tr>
<tr>
<td>6. SDS-PAGE analysis of <em>ada</em>&lt;sup&gt;-oqt&lt;/sup&gt; whole cell sonicates containing either GST-MGMT or GST-Ada fusion proteins</td>
<td>53</td>
</tr>
<tr>
<td>7. SDS-PAGE analysis of <em>ada</em>&lt;sup&gt;-oqt&lt;/sup&gt; whole cell sonicates containing either MGMT-C35, MGMT-N18, or MGMT-N78 fusion proteins</td>
<td>54</td>
</tr>
<tr>
<td>8. SDS-PAGE analysis of <em>ada</em>&lt;sup&gt;-oqt&lt;/sup&gt; whole cell sonicates containing either ADA-PCHRV-MGMT, MGMT-PCHRV-ADA, or ADA-MGTTTAIL fusion proteins</td>
<td>55</td>
</tr>
<tr>
<td>9. SDS-PAGE analysis of <em>ada</em>&lt;sup&gt;-oqt&lt;/sup&gt; whole cell sonicates containing MGMT and Ada chimeric fusion proteins</td>
<td>57</td>
</tr>
<tr>
<td>10. SDS-PAGE analysis of GST-affinity purified MGMT and Ada GST-fusion proteins</td>
<td>58</td>
</tr>
<tr>
<td>11. SDS-PAGE analysis of GST-affinity purified ADA-PCHRV-MGMT and MGMT-PCHRV-ADA GST-fusion proteins</td>
<td>59</td>
</tr>
</tbody>
</table>
Figure 12. SDS-PAGE analysis of GST-affinity purified MGMT-ADA-MGMT GST-fusion proteins ... 61

13. Repair of O\textsuperscript{6}-MG in DNA by control and truncated MGMT and Ada fusion proteins ... 63

14. Quantitation of control and mutant MGMT fusion proteins ........ 66

15. Effect of 28 aa carboxyl-terminal tail deletion on the rate of MGMT-mediated repair of O\textsuperscript{6}-MG-containing DNA at 4\textdegree ... 68

16. Analysis of the temperature sensitivity of the MGMT-C28 mutant fusion protein ... 70

17. Effect of 28 aa carboxyl-terminal tail deletion on the rate of MGMT-mediated repair of O\textsuperscript{6}-MG-containing DNA at 37\textdegree C ... 71

18. Effect of 28 aa carboxyl-terminal tail deletion on the rate of MGMT-mediated repair of O\textsuperscript{6}-BG .......... 73

19. Analysis of repair of O\textsuperscript{6}-BG by the ADA-MGMTTAIL fusion protein ... 76

20. Repair of O\textsuperscript{6}-MG in DNA by control and truncated MGMT fusion proteins and oligopeptides ........ 77

21. Repair of O\textsuperscript{6}-MG in DNA by control MGMT, Ada, or MGMT-PCHRV-ADA, and ADA-PCHRV-MGMT fusion proteins ... 84

22. Repair of O\textsuperscript{6}-MG in DNA by ADA-LELS-MGMT chimeric fusion protein ........ 86

23. Repair of O\textsuperscript{6}-MG in DNA by MGMT-GLHE-ADA chimeric fusion protein ........ 87

24. Analysis of repair of O\textsuperscript{6}-BG by the MGMT-PCHRV-ADA and ADA-PCHRV-MGMT chimeric fusion proteins ........ 90

25. Analysis of repair of O\textsuperscript{6}-BG by the ADA-LELS-MGMT and MGMT-GLHE-ADA chimeric fusion proteins ........ 92

viii
<table>
<thead>
<tr>
<th>Figure</th>
<th>Summary diagram</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>26.</td>
<td>of the construction of MGMT-ADA-MGMT chimeric proteins</td>
<td>94</td>
</tr>
<tr>
<td>27.</td>
<td>Repair of O⁶-MG in DNA and O⁶-BG by control MGMT, Ada, and MGMT-ADA-MGMT chimeric fusion proteins</td>
<td>95</td>
</tr>
<tr>
<td>28.</td>
<td>Summary diagram of MGMT aa regions potentially involved in MGMT-mediated repair of O⁶-BG</td>
<td>97</td>
</tr>
<tr>
<td>29.</td>
<td>Repair of O⁶-MG in DNA by control MGMT, Ada, ADA-PCHRV-MGMT, or MGMT-PCHRV-ADA fusion proteins</td>
<td>101</td>
</tr>
</tbody>
</table>
**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Analysis of O⁶-BG repair activity by control MGMT, Ada, or truncated MGMT fusion proteins and oligopeptides</td>
<td>80</td>
</tr>
<tr>
<td>2.</td>
<td>Repair of O⁴-MT in DNA by MGMT-PCHRV-ADA and ADA-PCHRV-MGMT chimeric fusion proteins</td>
<td>100</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

A      adenine
aa     amino acid
Asn    asparagine
bp     base pair
BSA    bovine serum albumin
c      carboxyl
c      cysteine
cDNA   complementary deoxyribonucleic acid
CENU   chloroethylnitrosourea(s)
Ci     Curie
DNA    deoxyribonucleic acid
dNTP   deoxynucleotide triphosphate
dsDNA  double stranded deoxyribonucleic acid
DTT    dithiothreitol
EDTA   ethylenediamine tetraacetic acid
g      gram
Glu    glutamine
GST    glutathione S-transferase
h      hour(s)
HEPES  N-[2-hydroxyethyl]piperazine N-[2-ethansulfonic acid]
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>histidine</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HTH</td>
<td>helix-turn-helix</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thio-galactosidase</td>
</tr>
<tr>
<td>kb</td>
<td>kilobases</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-Morpholino) Ethane Sulfonic Acid</td>
</tr>
<tr>
<td>MGMT</td>
<td>O-6-methylguanine DNA methyltransferase</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>MNNG</td>
<td>N-methyl-N-nitro-N-nitrosoguanidine</td>
</tr>
<tr>
<td>MNU</td>
<td>N-methyl-N-nitrosourea</td>
</tr>
<tr>
<td>mol</td>
<td>mole(s)</td>
</tr>
<tr>
<td>N</td>
<td>amino</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>O⁶-BG</td>
<td>O⁶-benzylguanine</td>
</tr>
<tr>
<td>O⁶-MG</td>
<td>O⁶-methylguanine</td>
</tr>
<tr>
<td>O⁶-MT</td>
<td>O⁶-methylthymine</td>
</tr>
<tr>
<td>O.D.</td>
<td>optical density</td>
</tr>
<tr>
<td>P</td>
<td>proline</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>R</td>
<td>arginine</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>s</td>
<td>second(s)</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>TBE</td>
<td>tris-boric acid-EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>tris-EDTA</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>U</td>
<td>units</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>v</td>
<td>volts</td>
</tr>
<tr>
<td>V</td>
<td>valine</td>
</tr>
<tr>
<td>vol</td>
<td>volumes</td>
</tr>
<tr>
<td>X g</td>
<td>times gravity</td>
</tr>
</tbody>
</table>
O6-methylguanine DNA methyltransferase (MGMT) is a human DNA repair protein which removes O6-methyl and O6-alkyl groups from the O6-position of guanine in DNA. Since these methyl and alkyl lesions are believed to be associated with carcinogenesis induced by a variety of environmental mutagens, as well as with cytotoxicity caused by chemotherapeutic chloroethylnitrosoureas, the study of MGMT protein structure and function are of significant value in prevention and treatment of the tumorigenic state.

MGMT protein structure and function are best understood when compared to the Escherichia coli alkyltransferase, Ada. The human MGMT and E. coli Ada repair DNA adducts through a common mechanism involving the stoichiometric transfer of chloroethyl or alkyl groups from the O6-position of guanine to a cysteine contained within the protein’s highly conserved proline-cysteine-histidine-arginine-valine (PCHR) acceptor
Despite the common repair mechanism among MGMT and Ada, these proteins exhibit differential substrate specificities. Specifically, only MGMT repairs the bulky lesion, O6-benzylguanine (O6-BG) and only Ada efficiently repairs O4-methylthymine (O4-MT) in DNA. It remains unclear, however, what divergent aa outside the PCHRV acceptor site discriminate between O4-MT in DNA and O6-BG. Additionally, the contribution of aa in alkyltransferase-mediated DNA repair is relatively unknown. The studies in this dissertation were therefore designed to systematically analyze the role of aa regions in alkyltransferase activity and substrate specificity by generating a series of full-length, truncated, and chimeric MGMT and Ada GST-fusion proteins, and MGMT oligopeptides, and assessing the ability of the wildtype and mutant proteins to repair O6-methylguanine (O6-MG) and O4-MT in DNA and O6-BG.

With regard to protein activity, aa located throughout nearly the entire protein are involved in MGMT-mediated repair of O6-MG in DNA. The unique MGMT 28 aa carboxyl-terminal tail, however, was not required for the repair of O6-MG in DNA or O6-BG, although deletion of the tail reversibly inhibited the ability of MGMT to repair O6-MG in DNA at 4°C. This carboxyl-terminal tail, while affecting activity at lower temperatures, may also comprise a separate functional domain that mediates non-repair related activities.
With regard to substrate specificity, the unique ability of MGMT to interact with 0'-BG appears to reside in aa located amino-terminal to the PCHRV acceptor site while aa involved in Ada-selective repair of O'-MT are located both amino- and carboxyl-terminal to the PCHRV acceptor site. These results suggest that different aa regions in MGMT and Ada define the overall substrate specificity of the proteins.
CHAPTER 1
INTRODUCTION

Cancer is a disease in which normal cell growth becomes deregulated, resulting in tumor formation. Human tumors usually develop in progressive stages, resulting finally in an invasive, metastasizing, malignant cancer (Cairns, 1985). Metastatic spread of malignancy is the primary cause of treatment failure and subsequent death in cancer patients (Cairns, 1985). Multiple epigenetic and environmental factors that interact over long periods of time are involved in the generation of a malignant cell suggesting that carcinogenesis is a multicausal, multistep process.

Cytotoxic chemotherapy is one of the most effective treatments against a wide variety of human cancers. This type of chemotherapy involves the use of alkylating agents such as the chloroethylnitrosoureas (CENU). Chloroethylnitrosoureas constitute a clinically important group of chemotherapeutic alkylating agents that damage DNA at nucleophilic sites including the O6-position of guanine (Tong et al., 1983). The O6-chloroethylguanine adduct is believed to mediate the cytotoxic potential of CENU as this adduct undergoes a slow intramolecular rearrangement to ultimately yield N-1 guanine, N-3 cytosine DNA interstrand
crosslinks that are lethal to rapidly dividing tumor cells (Brent, 1985).

The clinical utility of CENU as anti-cancer agents is limited due to the ability of approximately 80% of human tumor cell lines to repair the CENU-induced chloroethyl group adduct at the O\textsuperscript{6}-position of guanine in DNA thus preventing the formation of cytotoxic DNA interstrand crosslinks (Erickson et al., 1980a; Robins et al., 1983; Pegg and Dolan, 1987; Smith and Brent, 1989). This resistance is conferred by a human DNA repair enzyme, O\textsuperscript{6}-methylguanine DNA methyltransferase (MGMT) (Pegg et al., 1982; Tano et al., 1990; Mitra and Kaina, 1993). MGMT irreversibly transfers chloroethyl and alkyl group adducts from the O\textsuperscript{6} position of guanine in DNA to a cysteine acceptor site contained within it’s primary structure (Pegg et al., 1983; von Wrons ̈ki et al., 1991). This transfer returns the damaged DNA base to an unmodified state and permanently inactivates the protein (Mehta et al., 1981; Pegg et al., 1982). While most normal human tissues contain MGMT repair activity, approximately 20% of human tumor cell lines are deficient in MGMT activity and are thus much more sensitive to CENU-induced cytotoxicity (Day et al., 1980; D'Incalci et al., 1988). Studies developing enhanced cancer chemotherapy regimens, therefore, have been directed at depleting MGMT activity in MGMT-expressing tumor cell lines. MGMT depletion strategies have involved the use of non-cytotoxic MGMT inhibitors such as O\textsuperscript{6}-
methylguanine (O\textsuperscript{6}-MG) or the free base guanine analogue, O\textsuperscript{6}-benzylguanine (O\textsuperscript{6}-BG), prior to CENU therapy. Since O\textsuperscript{6}-MG and O\textsuperscript{6}-BG directly interact with and inactivate MGMT, these compounds have proven in the laboratory setting to enhance the cytotoxic potential of CENU in MGMT-expressing tumors (Dolan et al., 1985; Dolan et al., 1989; Dolan et al., 1990).

It should be noted, however, that these compounds, while somewhat effective, may also increase the dose-limiting toxicity of CENU to the bone marrow (DeVita et al., 1965; Gerson et al., 1987). This problem might be eliminated, however, by designing strategies involving transfection of bone marrow cells with genetically engineered inhibitor-insensitive MGMT proteins preceding CENU-MGMT inhibitor therapy. The protection of normal but not tumor cells from CENU-induced cytotoxicity would provide a more effective chemotherapeutic strategy. The development of these proposed regimens, however, relies on the design of better MGMT inhibitors and novel MGMT proteins with altered substrate specificities, which in turn depends upon a better understanding of how the MGMT protein interacts with its DNA substrates.

MGMT protein structure and function are best understood when compared to the *Escherichia coli* alkyltransferase, Ada. The human MGMT and *E. coli* Ada repair DNA through a common mechanism involving the transfer of chloroethyl or alkyl groups from the O\textsuperscript{6}-position of guanine to a cysteine
contained within the protein's conserved proline-cysteine-histidine-arginine-valine (PCHRV) acceptor site (Pegg and Byers, 1992). These five invariant aa are believed to be required for protein activity (Ling-Ling et al., 1992; Pegg, 1990) while the contribution of aa outside the acceptor site in alkyltransferase-mediated O⁶-alkylguanine repair is relatively unknown. Since MGMT and Ada share a high degree of aa sequence homology surrounding the PCHRV acceptor site, the possibility exists that these conserved sequences contribute to and are neccessary for alkyltransferase function. Although MGMT and Ada exhibit similar characteristics, these proteins also have different substrate specificities. Specifically, only MGMT repairs the bulky lesion, O⁶-BG (Dolan et al., 1991; Pegg et al., 1993), and only Ada efficiently repairs O⁴-MT in DNA (Dolan et al., 1984; McCarthy et al., 1984). It remains unclear, however, what divergent aa outside the PCHRV acceptor site discriminate between O⁴-MT in DNA and O⁶-BG. In considering the aa that may play a role in both alkyltransferase activity and substrate specificity, a series of mutant MGMT and Ada proteins were generated and assessed for their ability to repair O⁶-MG and O⁴-MT in DNA and the free damaged base O⁶-BG.

The purpose of my dissertation research is to determine the relationship between MGMT protein structure and function. I have proposed two specific aims to examine this relationship:
Aim 1: To determine the role of human MGMT sequences other than the cysteine acceptor site in the repair of O\textsuperscript{6}-methylguanine adducts in DNA.

Aim 2: To identify human MGMT sequences that play a role in substrate specificity.

The significance of the proposed studies is two fold. First, the identification of aa which contribute to MGMT activity and substrate specificity will contribute to an understanding of how MGMT functions. Second, identification of such functionally relevant regions of the protein could guide development of better MGMT inhibitors and therapeutically useful MGMT proteins which may ultimately help overcome the resistance of MGMT-expressing tumor cells to CENU, thus enhancing cancer chemotherapy.
A. DNA Alkylating Agents

Alkylating agents include potent environmental mutagens and carcinogens as well as anti-tumor compounds (Maher et al., 1990). These agents can react with nucleophilic groups on all four bases of DNA such as the exocyclic oxygens and endocyclic nitrogens as well as the sugar phosphates of the DNA backbone (Loveless, 1969; Singer, 1976). The distribution of these adducts at various sites within DNA depends on the chemical structure of the alkylating agent and the alkyl group itself. DNA adducts arising from alkylating agents are relevant to humans mainly because of the widespread exposure to nitrosoamines in our diet (Lyon, 1978), continuous environmental exposure to alkylating agents including tobacco smoke (Wynder and Hoffman, 1967), and the clinical utility of alkylating agents in cancer chemotherapy.

Monofunctional DNA alkylating agents are some of the most potent and widespread mutagens and carcinogens known and include such compounds as N-methyl-N-nitro-N-nitrosoguanidine (MNNG), N-methyl-N-nitrosourea (MNU) and dimethylnitrosamine.
These reactive compounds alkylate DNA to form a variety of adducts, predominantly methylations at the N\textsuperscript{7}-position of guanine, the N\textsuperscript{3}-position of adenine, the O\textsuperscript{6}-position of guanine, and phosphodiester oxygens (Lawley, 1976; Singer, 1979). These compounds can also react with DNA to form lesser amounts of methylated pyrimidine nitrogens and oxygens including methylations at the O\textsuperscript{2}-position of thymine, the O\textsuperscript{4}-position of thymine, and the O\textsuperscript{2}-position of cytosine (Lawley, 1976; Singer, 1979). Although all of these DNA lesions are a potential source of mutation, numerous reports have indicated that alkylation at the O\textsuperscript{6}-position of guanine, and to a lesser extent the O\textsuperscript{4}-position of thymine, by these environmental agents is of particular importance for the induction of mutations and cancers (Loveless, 1969; Toorchen and Topal, 1983; Day et al., 1987; Day and Ullah, 1992). During DNA replication, O\textsuperscript{4}-MT mispairs with guanine, resulting in a T:A to C:G transition (Preston et al., 1986), while O\textsuperscript{6}-MG preferentially pairs with thymine rather than cytosine resulting in a G:C to A:T transition mutation (Snow et al., 1984; Loechler et al., 1984; Preston et al., 1986). These mutagenic modifications can be lethal depending on whether the modification occurs at a site essential or non-essential for protein or nucleic acid structure or function (Kumar et al., 1990). Some of these mutations may result in the activation of oncogenes thus inducing malignant transformation of cells (Kumar et al., 1990). In particular,
studies have found that activation of the proto-oncogenes Ha-ras or K-ras in rat mammary and esophageal tumors or mouse lung tumors induced by MNU occurs by means of a G:C to A:T transition at the guanine in the second base of codon 12 (Sukumar et al., 1983; Zarbl et al., 1985; Inui et al., 1994), consistent with the formation and persistence of O\textsuperscript{6}-MG at this site.

The cytotoxicity of environmental alkylating agents is also attributed in part to the accumulation of O\textsuperscript{6}-MG pre-mutagenic mispairings in DNA. Such accumulation of O\textsuperscript{6}-MG lesions is believed to cause abortive mismatch repair (Day and Ullah, 1992; Karran and Bignami, 1992; Branch et al., 1993) and abnormal induction of intra-chromosomal homologous recombination thus leading to chromosomal aberrations (Maher et al., 1990; Rasouli-Nia et al., 1994). In the mismatch repair model, the O\textsuperscript{6}-MG:T pair is recognized by the mismatch correction system which initiates excision of the incorrect thymine residue. It has been suggested by Karran et al., 1994, that the subsequent repair synthesis, which occurs in the strand opposite of the O\textsuperscript{6}-MG, inevitably fails owing to the inability of the repair system to find a perfect complementary match for O\textsuperscript{6}-MG. Thus, the mismatch repair system may initiate an abortive correction that results in a repair patch in the strand opposite O\textsuperscript{6}-MG (Karran and Bignami, 1992). Persistence of the O\textsuperscript{6}-MG lesions coupled with abortive mismatch repair may result in elevated DNA
strand breaks. DNA strand breaks persisting into the subsequent cell cycle can inhibit the initiation of DNA replication and are linked to cell killing (Singer, 1979).

In contrast to environmental DNA alkylating agents, bifunctional chemotherapeutic alkylating agents such as chloroethylnitrosoureas (CENU) are clinically important in the treatment of cancer. CENU are used singly and in combination with other chemotherapeutic agents in the therapy of a wide variety of human neoplasias including lymphomas, colon, lung, and brain cancers, and drug-resistant multiple myeloma (Salmon, 1976; D'Incalci et al., 1988). CENU spontaneously decompose under physiologic conditions to form a chloroethylating moiety and a carbamoylating moiety (Montgomery et al., 1967; Colvin et al., 1976). The chloroethylating moiety can alkylate DNA at numerous nucleophilic sites including the O6-position of guanine (Tong et al., 1983). The O6-chloroethyl group adducts on guanine are believed to be the lesions most relevant to the cytotoxicity induced by CENU (reviewed in Erickson, 1991) as these adducts undergo a slow intramolecular rearrangement to form initially a cyclized intermediate, O6-N1-ethanoguanine, and ultimately, through interaction with the cytosine on the complementary DNA strand, an N1-guanine, N3-cytosine DNA interstrand crosslink (Brent, 1985). DNA interstrand crosslink formation induced by CENU correlates well with the inhibition of DNA replication and RNA transcription.
(Friedberg, 1985), the induction of G2 arrest, and subsequent tumor cell death (Gralla et al., 1987).

B. **O⁶-Methylguanine DNA Methyltransferase (MGMT)**

The human DNA repair protein, O⁶-methylguanine-DNA methyltransferase (MGMT), by means of its ability to repair CENU-induced O⁶-guanine monoadducts prior to DNA interstrand crosslink formation, represents the primary means of cellular resistance to the cytotoxic effects of CENU (Erickson et al., 1980; Pegg and Dolan, 1987; Smith and Brent, 1989). MGMT, acting as a 22 kDa monomer, stoichiometrically transfers alkyl and chloroethyl group adducts from the O⁶-position of guanine in DNA to a cysteine residue in its structure, thereby repairing DNA lesions in a single step reaction (Pegg and Byers, 1992; Mitra and Kaina, 1993). The transfer reaction is irreversible, forming S-methylcysteine in the protein and undamaged guanine in the DNA substrate (Mehta et al., 1981; Pegg et al., 1982). MGMT can also form covalent interactions with the CENU-induced cyclized intermediate O⁶-N¹-ethanoguanine (Gonzaga et al., 1992). The number of O⁶-alkylguanine lesions that can be repaired is directly proportional to the number of intracellular MGMT molecules. While most normal human tissues contain MGMT repair activity, approximately 20% of human tumor cell lines are deficient in MGMT activity and are thus much more sensitive to CENU-
induced cytotoxicity (Day et al., 1980; Erickson et al., 1980a; D'Incalci et al., 1988). Cell lines deficient in MGMT become resistant, however, to the cytotoxic effects of alkylating agents when a bacterial methyltransferase gene (ada) or the MGMT cDNA is transfected and appropriately expressed (Kataoka et al., 1986; Samson et al., 1986). These findings and others (Ewing and Kohn, 1978; Erickson et al., 1980; Erickson et al., 1980a) establish a dominant role for MGMT in conferring CENU resistance and, in combination with other studies (Futscher et al., 1989; Dolan et al., 1990; Marathi et al., 1993), demonstrate the usefulness of inhibiting MGMT for therapeutic benefit.

The cloning of the human MGMT cDNA (Tano et al., 1990; Hayakawa et al., 1990; Rydberg et al., 1990) has offered the opportunity to express and isolate MGMT in sufficient amounts for detailed biochemical analyses. The MGMT cDNA was first isolated by transforming alkyltransferase deficient bacteria with a cDNA library made from an MGMT-expressing cell line, followed by phenotypic rescue of repair proficient cells in media containing MNNG (Tano et al., 1990). The protein sequence encoded by the MGMT cDNA consists of 207 aa and has a predicted molecular weight of about 22 kDa (Bhattacharyya et al., 1990). There is no evidence of a nuclear localization sequence although MGMT has been found to be localized exclusively in the nucleus (Ayi et al., 1992; Ishibashi et al., 1994) and is chromatin-bound (Boulden et
al., 1987). MGMT requires no post-translational modifications or cofactors, has an optimal pH of approximately 7.7-8.5 (Pegg et al., 1983; Boulden et al., 1987), and is strongly inhibited by high salt concentrations (Bhattacharyya et al., 1990) and metal ions (Scicchitano and Pegg, 1987). In addition to the human MGMT, alkyltransferases from yeast (Xiao et al., 1991), rat (Potter et al., 1991), mouse (Santibanez-Koref et al., 1992), hamster (Rafferty et al., 1992), rabbit (Iyama et al., 1994), E. coli (Ada, ogt) (Demple et al., 1985; Potter et al., 1987) and Bacillus subtilis (dat) (Kodama et al., 1989) have all been isolated. The E. coli Ada protein, as discussed below, was the first to be isolated and extensively characterized (Demple et al., 1985).

C. *Escherichia coli* Alkyltransferase (Ada)

The 39 kDa Ada protein contains two independent DNA repair domains separated by a protease-sensitive hinge region (Jeggo, 1979; Demple et al., 1982). The 39 kDa Ada protein can be proteolytically cleaved at this hinge region (located between lysine-178 and glutamine-179) (Teo et al., 1984; Demple, 1990) to yield a 19 kDa carboxyl-terminal fragment which repairs $O^6$-alkylguanine and $O^6$-alkylthymine adducts, and an amino-terminal fragment containing a second cysteine acceptor site which repairs alkylphosphotriesters (Demple and
It is the repair of alkylphosphotriesters by the amino-terminal domain that converts the 39 kDa alkyltransferase into a strong activator of ada gene transcription by enhanced binding of the 39 kDa protein to the ada gene promoter region (Nakabeppu and Sekiguchi, 1986). This phenomenon, known as the adaptive response (which can also be down-regulated by proteolytic cleavage of the hinge region, Akimaru et al., 1990), provides an efficient system that allows bacteria to respond to DNA damage (Demple, 1986; Lindahl and Sedgwick, 1988; Shevell et al., 1990) caused by a wide variety of alkylating agents including endogenously produced N-nitroso compounds (Tsimis and Yarosh, 1990; Vaughan et al., 1991). Ada, therefore, not only repairs alkylated DNA bases and DNA phosphotriesters but also plays a role in transcriptional regulation of the ada gene.

In addition to the Ada protein, E. coli also contains a smaller methyltransferase encoded by the ogt gene. The 19 kDa ogt protein repairs O6-alkylguanine and O4-alkylthymine lesions and shares limited aa sequence homology to the carboxyl-terminal domain of the Ada protein (Potter et al., 1987; Wilkinson et al., 1989). Unlike Ada, which is inducible in response to alkylating agents, the ogt gene product is constitutively expressed and, in un-induced bacterial cells, provides the majority of the alkyltransferase activity (Rebeck et al., 1988; Shevell et
al., 1988; Rebeck et al., 1989). The ogt protein thus serves to counteract low-level $O^6\text{-MG}$ production in the bacterial genome (Potter et al., 1987).

D. MGMT and Ada Protein Structure and Function

All of the prokaryotic and eukaryotic alkyltransferases described to date share a high degree of primary aa homology (Santibanez-Koref et al., 1992; Moore et al., 1994) (Figure 1) which suggests that the proteins not only may be structurally similar but may also share a common DNA repair mechanism. Such a common repair mechanism has been defined for all alkyltransferases in which DNA repair involves the transfer of alkyl groups from the $O^6\text{-position}$ of guanine (and, in the case of Ada, the $O^4\text{-position}$ of thymine) to a cysteine contained within the invariant proline-cysteine-histidine-arginine-valine (PCHRV) acceptor site. The importance of this cysteine in alkyltransferase function was initially demonstrated in studies in which MGMT and Ada were reacted with radiolabeled alkylated DNA. In each case, the cysteine within the PCHRV motif was the sole alkyl group acceptor (Demple et al., 1985; von Wronski et al., 1991). Additionally, in site-directed mutagenesis studies, replacement of the acceptor cysteine in Ada with histidine or alanine (Takano et al., 1988; Tano et al., 1989), or in MGMT
Figure 1. Alkyltransferase sequence alignment. Escherichia coli Ada (Demple et al., 1985), S. typhimurium adaC (Rebeck et al., 1989), B. subtiliss adaB (Moore et al., 1994), B. subtiliss datI (Kodama et al., 1989), E. coli ogt (Potter et al., 1987) mouse (Santibanez-Koref et al., 1992), rat (Potter et al., 1991), hamster (Rafferty et al., 1992), human (Tano et al., 1990), and yeast (Xiao et al., 1991) alkyltransferases share a high degree of aa sequence homology (bordered by two large arrows) surrounding the protein’s PCHRV acceptor site (bordered by two small arrows). Identical aa are typed in bold.
with any of ten different aa (Ling-Ling et al., 1992), resulted in an alkyltransferase-negative phenotype. Furthermore, when the cysteine and histidine positions were inverted within the PCHRV acceptor site, alkyltransferase function was lost. These studies indicate that the exact position of cysteine within the acceptor site is critical for alkyltransferase function. In addition to cysteine, three other aa (P, H, and V) within the PCHRV acceptor site have also been suggested to be important for activity (Ling-Ling et al., 1992; Pegg and Byers, 1992). The contribution of these aa to repair is unproven although studies suggest that proline may serve to bend the protein in such a way as to properly orient the acceptor cysteine while the histidine and valine may play roles in proper protein folding and stability (Ling-Ling et al., 1992). Amino acids outside the PCHRV acceptor site are also suggested to be important for repair activity as demonstrated by the presence of several invariant aa surrounding the cysteine acceptor site in all alkyltransferases (Figure 1) and by site-directed MGMT mutagenesis studies in which aa substitutions at some of these invariant aa, including asparagine 137 (Pieper et al., 1994) and glutamic acid 172 (Rafferty et al., 1994), resulted in loss of activity. The importance of the PCHRV acceptor site and other surrounding conserved aa in alkyltransferase repair has been recently highlighted by crystallographic analysis of the 19 kDa carboxyl-terminal fragment of Ada
(Moore et al., 1994). Studies on the Ada crystal structure suggest that a histidine residue within PCHRV interacts with the acceptor cysteine and serves as a proton acceptor, generating a reactive cysteine thiolate anion that sequentially attacks, removes, and accepts alkyl lesions from the O6-position of guanine in DNA. The formation and stabilization of this anion are believed to be facilitated by invariant aa contained within the highly conserved carboxyl-terminal 85 aa region surrounding the PCHRV acceptor site. All of these studies therefore suggest that an understanding of aa involved in MGMT activity requires an analysis of aa beyond those directly involved in alkyl group transfer.

In addition to sharing a common acceptor site and DNA repair mechanism, MGMT and Ada also share a strong preference for double stranded O6-MG-containing oligodeoxynucleotide substrates greater than 12 bp (Scicchitano et al., 1986). In vitro studies concerning the polynucleotide requirements for alkyltransferase-mediated O6-MG repair indicate that by far dsDNA in the B-conformation, as opposed to the Z-conformation, is the best substrate, whereas O6-MG-containing RNA is a much poorer substrate (Lindahl et al., 1982; Harris et al., 1983; Boiteaux and Laval, 1985; Pegg et al., 1988). With regard to the repair of various lengths of O6-MG-containing oligodeoxynucleotide substrates, the rates of Ada- and MGMT-mediated O6-MG removal increase with increasing length of the substrate (i.e. rate of repair of
dodecadexytnucleotide＞hexadeoxyynucleotide＞tetradexyynucleotide＞free base) (Scicchitano et al., 1986; Graves et al., 1987; Dolan et al., 1988a), suggesting that the overall primary structure of dsDNA plays a vital role in the recognition and DNA repair processes. The finding that DNA binding may be important for alkyltransferase function is supported by the fact that O⁶-MG as a free base is an approximately 10⁷-fold poorer substrate than O⁶-MG in duplex DNA (Dolan et al., 1985; Yarosh et al., 1986). These large differences in affinities are explained by circular dichroism and fluorescence analyses which examined Ada- and MGMT-DNA interactions (Takahaski et al., 1990; Chan et al., 1993). These studies suggested that MGMT and Ada are activated by a conformational change induced by binding of the protein to damaged DNA and that this binding to DNA is the rate determining step in the transfer of alkyl groups to MGMT or Ada (Takahaski et al., 1990; Chan et al., 1993). Specifically, Ada, which has a ten times higher DNA binding affinity (Takahaski et al., 1990), repairs O⁶-MG in DNA at a significantly faster rate than the human MGMT protein. On the other hand, the repair of radiolabeled O⁶-MG containing DNA by either Ada or MGMT was only slightly inhibited by the addition of a 1000-fold excess of non-alkylated or alkylated DNA, suggesting that DNA binding may only play a small role in alkyltransferase activity (Graves et al., 1989). Nonetheless, a variety of studies have demonstrated that
alkyltransferases do have the capacity for high affinity DNA-binding and will interact with DNA irrespective of the O⁶-MG content (Bhattacharyya, et al., 1990; Pegg, 1990), suggesting that the alkyl group accepting site may be distinct from a possible DNA binding domain. The region of the protein responsible for DNA binding, however, has not been defined although there exist several aa regions surrounding the cysteine acceptor site that are rich in basic amino acids and thus are believed to be involved in DNA interaction (Pegg and Byers, 1992).

**E. MGMT and Ada Substrate Specificity**

An understanding of the similarities in alkyltransferase structure and function, however, does not explain the dramatically different abilities of alkyltransferases to interact with various types of DNA lesions. One may understand how alkyltransferases interact with distinct lesions by comparing the substrate specificities of the human MGMT to that of the *E. coli* Ada. The substrate specificities of MGMT and Ada are reviewed below with respect to the protein's relative preference for different sized alkyl groups at the O⁶-position of guanine in DNA and the ability of the proteins to repair O⁶-BG and O⁴-MT.
1. Differential repair of various sized $\text{O}^6$-alkylguanine adducts by MGMT and Ada

While $\text{O}^6$-MG in DNA is the preferred substrate, MGMT and Ada can also repair larger-sized alkyl groups at the $\text{O}^6$-position of guanine but at rates of reaction that decrease dramatically as the alkyl group size increases (following the sequence: methyl $> \text{ethyl} > \text{n-propyl} > \text{n-butyl} >> \text{isopropyl}, \text{isobutyl}, \text{2-hydroxyethyl})$. The repair of branched chain groups is much slower than that of linear alkyl groups (Morimoto et al., 1985; Pegg et al., 1985; Graves et al., 1989). Two main differences exist between MGMT and Ada in the rates of repair of these various sized alkyl groups at the $\text{O}^6$-position of guanine in DNA. First, MGMT repairs $\text{O}^6$-MG in DNA at a much lower rate than Ada (Bhattacharrya et al., 1988). Second, the difference between the relative rates of repair for $\text{O}^6$-MG and longer alkyl groups is much greater for Ada (amounting to a 1000-fold difference) than for the MGMT protein (Morimoto et al., 1985; Pegg et al., 1985; Lindahl and Sedgwick, 1988; Demple, 1990; Pegg, 1990). These differences in repair rates for larger-sized alkyl groups for both MGMT and Ada could be due to steric constraints in which the larger substrates may prevent access of the cysteine acceptor site of the protein to the oxygen-linked carbon of guanine. Alternatively, Pegg and Byers (1992) suggest that the ethyl and larger groups on the $\text{O}^6$-position of guanine may
adopt a novel conformation and be directed into the major
 groove of DNA in such a way that impedes DNA repair. Bulky,
 branched-chain adducts such as $O^6$-isobutylguanine that
distort the DNA helix, and act as DNA replication blocks, are
believed to be removed by the excision repair pathway (Samson
et al., 1988; Pegg and Byers, 1992).

2. Differential repair of $O^4$-methylthymine and $O^6$-
benzylguanine by MGMT and Ada

One may further define how alkyltransferases interact
with distinct lesions by comparing the abilities of MGMT and
Ada to repair $O^4$-MT in DNA, and the free base $O^6$-BG. Ada
efficiently repairs $O^4$-MT in a dodecadeoxynucleotide
substrate, or in poly d(A)-d(T), whereas MGMT-mediated repair
of this lesion is absent (McCarthy et al., 1984; Dolan et
al., 1984). Several studies suggest, however, that if a
1000-fold molar excess of MGMT protein is used, repair of $O^4$-
MT-containing DNA in vitro is detectable (Koike et al., 1990;
Sassanfar et al., 1991). This repair, however, occurs more
slowly than MGMT-mediated repair of $O^6$-MG in DNA (Becker and
Montesano, 1985; Brent et al., 1988; Dolan et al., 1988).
The rate of repair of $O^4$-MT in DNA by MGMT is at least 10,000
times lower than the repair of $O^6$-MG in DNA (O'Toole et al.,
1993). The extremely poor reactivity of MGMT toward $O^4$-MT
makes it unlikely that this repair reaction occurs to any
significant extent in human cells exposed to alkylating
agents, and the general consensus from in vitro and in vivo studies has been that MGMT does not repair O\(^4\)-MT in DNA (Belinsky et al., 1986; Brent et al., 1988; Hall et al., 1990; Pegg and Byers, 1992). An alternative pathway for O\(^4\)-MT repair has been detected in mammalian cells, but this repair activity has not been well characterized (Wani et al., 1990). MGMT and Ada are further distinguished by their differential sensitivities to O\(^6\)-BG. Only MGMT repairs O\(^6\)-BG, a guanine analog which was developed as a non-cytotoxic MGMT depleting agent (Pegg, 1990; Dolan et al., 1991; Pegg et al., 1993). O\(^6\)-BG is an excellent substrate, serving as an effective inactivator of MGMT (Dolan et al., 1990). This potent agent has thus been used by several groups (Dolan et al., 1990; Marathi et al., 1993) to markedly enhance the cytotoxic effects of chloroethylating agents toward MGMT-expressing human tumor cells. As mentioned above, the rate of MGMT-mediated repair of DNA adducts decreases as the alkyl group size increases, indicating that steric constraints limit the reaction. In the case of O\(^6\)-BG, however, the steric hindrance is offset by the greater ability of the benzene ring to delocalize charge in the transition state for a displacement reaction (Dolan et al., 1990). The Ada protein, however, may be more sterically hindered by the benzene ring suggesting that subtle differences in protein conformation around the protein's active site may be critical in allowing the interaction between the alkyltransferase and
larger-sized substrates such as O\textsuperscript{6}-BG (Dolan et al., 1991).

It remains unclear which part of the protein, or which aa, discriminate between O\textsuperscript{4}-MT in DNA and O\textsuperscript{6}-BG. The basis for differential substrate specificities, however, clearly resides outside the protein's shared PCHRVR acceptor site, and may lie in subtle differences in the protein's primary aa sequence. Two major structural differences between MGMT and Ada include a highly divergent amino-terminal 75 aa segment, and a MGMT 28 aa carboxyl-terminal tail (Figure 2). This carboxyl-terminal extension is highly conserved among human, rat, mouse, and hamster alkyltransferases but is absent in yeast and bacterial alkyltransferases such as E. coli Ada, ogt and B. subtilis dat. Divergent aa or aa regions, while of unknown function, may contribute to the differences in MGMT-mediated repair of O\textsuperscript{6}-BG and Ada-mediated repair of O\textsuperscript{4}-MT.
Figure 2. Alkyltransferase sequence alignment. *Escherichia coli* Ada (Demple et al., 1985), *S. typhimurium* adaC (Rebeck et al., 1989), *B. subtiliss* adaB (Moore et al., 1994), *B. subtiliss* dat1 (Kodama et al., 1989), *E. coli* ogt (Potter et al., 1987) mouse (Santibanez-Koref et al., 1992), rat (Potter et al., 1991), hamster (Rafferty et al., 1992), human (Tano et al., 1990), and yeast (Xiao et al., 1991) alkyltransferases exhibit two main structural differences. These two differences include an amino-terminal 75 aa segment (bordered by two arrows) which is relatively non-conserved between proteins, and a 28 aa carboxyl-terminal tail (designated by solid lines) which is highly conserved only among mouse, rat, hamster, and human alkyltransferases. Identical aa are typed in bold. Alignment of the numbered aa is according to Moore et al., 1994.
CHAPTER III
MATERIALS AND METHODS

A. Bacteria and Plasmids

*E. coli* strain GWR111 [(*ada*-alkB), kan+, ogt-] was used as a methyltransferase deficient host cell and was provided by Dr. B. Demple (Rebeck and Samson, 1991). The plasmid, pGEX-3X (Pharmacia LKB; Uppsala, Sweden), was used as a prokaryotic expression vector. This plasmid contains the GST gene from *S. japonicum* which encodes a 26 kDa protein. The GST protein is fused to the protein products of coding sequences inserted into the multiple cloning site. Between the GST gene and the cDNA inserts, a recognition sequence for a site-specific protease (factor Xa) is encoded. The expression vector directs the synthesis of GST fusion proteins in *E. coli* under the control of the IPTG-inducible *tac* promoter.

B. Cell Culture

The HT-29 colon carcinoma cell line was obtained from Dr. L. Erickson and was cultured in Eagle's minimum essential
medium supplemented with 10% bovine calf serum, glutamine, sodium pyruvate, vitamin B12, nonessential amino acids, and gentamicin. Cultures were incubated at 37°C in a 95% air, 5% CO₂ atmosphere.

C. Generation of Full-Length, Truncated, and Chimeric MGMT and ada cDNAs by PCR Amplification

Plasmid DNA containing a 721bp human MGMT cDNA (Tano et al., 1990) or a 2.3kb ada-alkB operon (Demple et al., 1985) was digested with HindIII (10U/µg DNA, 2h, 37°C) and used as template in PCR amplification reactions. Full-length and truncated MGMT and ada cDNAs were generated using the following reaction conditions: DNA templates (1 µg) were added to a 100µl total volume solution containing 1X PCR buffer (10mM Tris-HCl pH 8.3, 50mM KCl, 2.5mM MgCl₂, 0.01% gelatin), and 200 µM each of dCTP, dATP, dGTP, and dTTP. To the mixture were added 50 pmol each of two synthetic oligonucleotides complementary either to the human MGMT cDNA or to the E. coli. ada DNA. Primers pr1 (5' - CGTGGGATCCCCCCATGGACAGGATTGTGA-3'), nterm18 (5' - CGTGGGATCCCCCCTGGAGCTGTCTGGTTG-3'), and nterm78 (5' - CGTGGGATCCCCCTTCCCCGTGCGGCTCTTCA-3') are complementary to different regions of the 5' end of the non-transcribed strand of the human MGMT cDNA. Each primer contains 12 non MGMT-complementary nt at its 5' end which encode a recognition sequence for BamH1. Primers pr2 (5' - CGATGAATTCTCAGTT
TCGGCCAGCAG-3'), cterm10 (5'-CGATGAATTCTCAAGCTCCGCCTCCGCTC
CCTTGAGCCA-3'), cterm28 (5'-CGATGAATTCTCACAAACCGGTGGGCTTCAT-
3'), cterm35 (5'-CGATGAATTCTCTCACTGGGAGGACAGAG-3'), and
cterm65 (5'-CGATGAATTCTCAACCGGTGGGACAGGATT-3') are
complementary to different regions of the 3' end of the
transcribed strand of the human MGMT cDNA. Each primer
contains 13 non MGMT-complementary nt at its 5' end which
encode a stop codon and an EcoR1 restriction site. Primer
adaup (5'-CGTGGGATCCCCATGCAATTCCGTCACGGTGGC-3') is
complementary to the 5' end of the nontranscribed strand of
the E. coli ada DNA sequence and contains 15 non-ada-
complementary nt which encode a recognition sequence for
BamH1. Primer adadn (5'-CGATGAATTCTTACCTCCTCATTTTCAG-3')
is complementary to the 3' end of the transcribed E. coli ada
DNA sequence and contains 13 non-ada-complementary nt which
encode a stop codon and an EcoRI restriction site. Taq DNA
polymerase (2U; Perkin Elmer Cetus, Corp., Norwalk, CT.) was
added to the PCR reaction and DNA amplification was performed
for 20 cycles in a DNA thermal cycler (Perkin Elmer Cetus;
denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and
extension at 72°C for 45 s for each cycle). After
amplification, 10 µl of each PCR reaction mixture were
fractionated by electrophoresis (75 V, 30 min) along with a
molecular weight marker in a 1% agarose gel (Seakem GTG, FMC,
Rockland, ME) to confirm the size of the amplification
products. Primers pr1-pr2 amplify a 645-bp cDNA sequence
which encodes a full-length MGMT protein (MGMT). Primers nterm18-pr2 amplify a 567-bp MGMT cDNA fragment which encodes a protein (MGMT-N18) lacking 18 aa from the amino-terminus. Primers nterm78-pr2 amplify a 387-bp MGMT cDNA fragment which encodes a protein (MGMT-N78) lacking 78 aa from the amino-terminus. Primers pr1-cterm10 amplify a 621-bp MGMT cDNA fragment which encodes a protein (MGMT-C10) which lacks 10 aa from the carboxyl-terminus. Primers pr1-cterm28 amplify a 549-bp MGMT cDNA fragment which encodes a protein (MGMT-C28) lacking the 28 aa carboxyl-terminal tail. Primers pr1 and cterm35 amplify a 516-bp cDNA sequence which encodes a protein (MGMT-C35) lacking 35 aa from the carboxyl-terminal end. Primers pr1-m2 amplify a 450 bp MGMT cDNA fragment which encodes a protein (MGMT-C65) lacking 65 aa (including the PCHRV acceptor site) from the carboxyl-terminus. Primers adaup-adadn amplify a 553-bp DNA fragment which encodes an active 19kDa alkyltransferase carboxyl-terminal domain (Ada) which is part of the larger 39kDa *E. coli* Ada protein.

All MGMT-ada, ada-MGMT, and MGMT-ada-MGMT chimeric cDNAs were generated using the PCR overlap extension technique. PCR amplification of each half of the MGMT-ada and ada-MGMT cDNAs was performed separately, followed by joining of the halves in a third PCR reaction (Ho et al., 1989). For generation of each half of MGMT-ada chimeric cDNAs, linearized plasmid containing MGMT or ada cDNAs was amplified using two primers complementary either to the MGMT cDNA or to
the ada DNA. MGMT cDNA complementary primers were prl (described above), chim3 (5′-TACCCGATGACAGGGGATGAGGATGGG-3′) or 3glhe (5′-ATCATCGCCCAACTTTATTTTCTGTCAG-3′). Ada DNA complementary primers were adadn (described above), chim4 (5′-CCCTGTCATCGGTTAGTACCGCTGGT-3′), or 5glhe (5′-TTGCTGGGGCATGATGACGACCCACATT-3′). Primers chim4 and 5glhe contain at their 5′ ends 15nt complementary to chim3 and 3glhe primers, respectively. For amplification of each half of ada-MGMT chimeric cDNAs, primers complementary either to the ada DNA or the MGMT cDNA were used. Ada DNA complementary primers were adaup (described above), chim1 (5′-TCCGCTGCTGCAGGCTACGCATGACAGG-3′), tailada (5′-CCTCCTCATTTCAGCTTG-3′) or 3lelsg (5′-ACCAGACAGCTCCAGACCCGAGGTAT-3′). MGMT cDNA complementary primers were pr2 (described above), chim2 (5′-GTCTGCAGCGAGCGGTGGGAGGG-3′), tailmgmt (5′-GCTGAAAATGAGGAGCCAGCTGGGAGGG-3′) or 5lelsg (5′-CTGGAGCTGTCTGGTTGTGAGCAGGGT-3′). Primers chim2, tailmgmt, and 5lelsg contain at their 5′ ends 15 nt complementary to chim1, tailada, and 3lelsg primers, respectively. For generation of the MGMT-ada-MGMT chimeric cDNAs, overlapping MGMT-ada and ada-MGMT cDNA fragments were PCR amplified separately followed by joining of the halves in a third PCR reaction. For generation of the first half of MGMT-ada-MGMT cDNAs (MGMT-ada cDNA fragment), linearized plasmid containing MGMT cDNA was amplified using two primers complementary to the MGMT cDNA. Primers used were prl
For generation of the second half of MGMT-ada-MGMT cDNAs (ada-MGMT cDNA fragment), linearized plasmid containing MGMT cDNA was amplified using two primers complementary to the MGMT cDNA. Primers used were 5-reg1 (5'-GCTAACGCCAT CCGCAAACCGAAAGCGGTACGGGCGTTGCAAATGGCCTGTTGCGCCAACC CTGTCCCCATCCTC-3'), 5-reg2 (5'-TGGCAGGCACTGCGCACGATACCTTG CGGTGAAACGGTACGTTATCAGCAACTGGCAGCCCTGGCAGGC-3'), 5-reg3 (5'-CCGCTGGACATTCGCGGCACTGCTTTTCAGCAAGAGTCGTTCACCAGACACG-3'), 5-reg4 (5'-GAAGTGATCGGCGTACGGTACCTCAATCAACCGGATACCCCGCGCTGTTCCC CGTGCCGGCTCTT-3'), 5-reg5 (5'TTGCTGGGCAGATGAGGGTACGAGGCCACA TTAGATGGCGTGAGGGTC-3'), or pr2 (described above). Primers 3-reg1, 3-reg2, 3-reg3, 3-reg4, and 3-reg5 contain at their 5' ends ada nt complementary to 5-reg1, 5-reg2, 5-reg3, 5-reg4, and 5-reg5 primers, respectively. The conditions for the PCR amplification reaction were as described above. Following amplification, 80µl of each PCR reaction mixture were fractionated by electrophoresis (100v, 1hr) along with a molecular weight marker in a 1% agarose gel to confirm the size of the amplification products. Each PCR-amplified DNA fragment was excised from the agarose gel and purified (Geneclean II, Bio101 Inc., LaJolla, CA.).
generated, purified cDNA fragments (1µg) were then used in a subsequent PCR overlap extension reaction. Aliquots from two separate PCR reactions containing the overlapping fragments were mixed and subjected to a second PCR amplification using a pair of external synthetic oligonucleotides prl and adadn (amplified MGMT-ada cDNAs), adaup and pr2 (amplified ada-MGMT cDNAs), or prl and pr2 (amplified MGMT-ada-MGMT cDNAs). The reaction conditions used for the fusion of the two PCR-generated cDNA fragments were identical to those described above. Following amplification the size of the products were confirmed by agarose gel electrophoresis. Two sets of primers, prl-chim3 and adadn-chim4 were used to construct a 553-bp cDNA fragment which encodes an MGMT-PCHRV-ADA chimera fused at the PCHRV acceptor site. Primers prl-3glhe and adadn-5glhe were used to construct a 550-bp cDNA fragment which encodes a protein fused at the MGMT glycine-leucine-histidine-glutamic acid (GLHE) sequence (MGMT-GLHE-ADA). Primers adaup-chim1 and pr2-chim2 were used to construct a 630-bp cDNA fragment which encodes an ADA-PCHRV-MGMT chimera fused at the PCHRV acceptor site. Primers adaup-3lelsg and pr2-5lelsg were used to construct a 640-bp cDNA fragment which encodes a protein fused at the MGMT leucine-glutamic acid-leucine-serine (LELS) sequence (ADA-LELS-MGMT). Primers adaup-tailada and pr2-tailmgmt were used to construct a 637-bp cDNA fragment which encodes an Ada protein (ADA-MGTTAIL) fused to the MGMT carboxyl-terminal tail. Primers prl-3regl
and 5reg1-pr2 were used to construct a 645-bp cDNA fragment which encodes a chimeric protein containing Ada aa 118-137 (MGMT-ADA1-MGMT). Primers pr1-3reg2 and 5reg2-pr2 were used to construct a 645-bp cDNA fragment which encodes a chimeric protein containing Ada aa 99-117 (MGMT-ADA2-MGMT). Primers pr1-3reg3 and 5reg3-pr2 were used to construct a 642-bp cDNA fragment which encodes a chimeric protein containing Ada aa 79-92 (MGMT-ADA3-MGMT). Primers pr1-3reg4 and 5-reg4-pr2 were used to construct a 645-bp cDNA fragment which encodes a chimeric protein containing Ada aa 66-78 (MGMT-ADA4-MGMT). Primers pr1-3reg5 and 5reg5-pr2 were used to construct a 642-bp cDNA fragment which encodes a chimeric protein containing Ada aa 32-41 (MGMT-ADA5-MGMT).

In addition to the generation of the full-length, truncated and chimeric MGMT and ada cDNAs, four synthetic, MGMT acceptor site-containing oligopeptides (Peptide 1= PCHRV, Peptide 2= GNPVPILIPCHRVVCSSGAV, Peptide 3= AMRGNPVPILIPCHRV, and Peptide 4= PCHRVVCSSGAVGNYS) (Tano et al., 1990) were commercially synthesized and HPLC purified (Chiron Mimotopes). The oligopeptides were approximately 68-75% pure.
D. Preparation of Competent Alkytransferase Deficient \textit{E. coli} Cells

A single colony of \textit{E. coli} ada-ogt- cells was aseptically inoculated into 50 ml LB media containing ampicillin (100\(\mu\)g/ml) and the bacterial culture was grown overnight at 37\(^\circ\)C in a shaking incubator (250 rpm). The overnight culture was diluted (1:100 in LB broth) and grown as described above to an O.D._{590} of 0.3 to 0.4. The bacterial culture was aliquoted into prechilled, sterile polypropylene tubes and cooled on ice for 10 min. The bacterial cells were pelleted (1,600 g, 7 min, 4\(^\circ\)C), resuspended in 10 ml ice-cold CaCl\(_2\) solution (60mM CaCl\(_2\), 15\% glycerol, 10mM PIPES, pH 7.0), and repelleted (1,100 g, 5 min, 4\(^\circ\)C). The bacterial cells were resuspended in 10 ml ice-cold CaCl\(_2\) solution and left on ice for 30 min. The competent bacterial cells were pelleted (1,100 g, 5 min, 4\(^\circ\)C), resuspended in 2 ml of ice-cold CaCl\(_2\) solution, aliquoted into prechilled microcentrifuge tubes, and stored at -80\(^\circ\)C.

E. Subcloning of MGMT and ada cDNAs into a Bacterial Expression Vector

Ten \(\mu\)g of PCR products and the 4.9kb pGEX-3X bacterial expression vector (50 \(\mu\)g; Pharmacia LKB) were digested sequentially with BamH1 and EcoR1 (5U/\(\mu\)g, 37\(^\circ\)C, 2 h). Between digests, the DNA was extracted (once with an equal volume phenol /24:1 chloroform-isoamyl alcohol, and once with
an equal volume 24:1 chloroform-isoamyl alcohol), ethanol precipitated (0.1 vol 3M sodium acetate, 2 vol ethanol, 1 h, -80°C) and pelleted (12,000 g, 30 min, 4°C). The DNA pellet was dried under vacuum and resuspended in a buffer containing 10mM Tris-HCl and 1mM EDTA, pH 8.0. Digestion products were fractionated by electrophoresis in a 1.0% agarose gel at 20 V for 16 h. The PCR products were excised from the agarose gel, purified (Geneclean II, BIO 101 Inc.) and ligated (14°C, 16 h, 1U T4 DNA ligase) separately into a BamHI-EcoRI digested pGEX-3X plasmid (3:1 molar ratio of insert:vector DNA). The ligation reaction was diluted 1:5 in deionized H2O and used to transform competent E. coli ada-ogt-cells. Transformed ada-ogt- cells were plated onto LB agar plates containing 100 µg/ml ampicillin and were incubated at 37°C for 16 h. Bacterial colonies were randomly chosen from each plate, grown in culture (100 ml LB broth, 100µg/ml ampicillin) and used for plasmid DNA isolation (Wizard Minipreps DNA purification system; Promega, Madison, WI.). The identity of the subcloned DNA was confirmed by plasmid DNA (1µg) digestion with BamHI and EcoRI (10U/µg, 37°C, 2 h). DNA sequencing (Taq Track, Promega, Inc., Madison, WI.) was performed to verify the 5' and 3' MGMT mutations, the absence of any PCR-induced point mutations within the MGMT cDNA encoding MGMT-C28, and the junction sites of all the MGMT-ada, ada-MGMT, and MGMT-ada-MGMT chimeric cDNAs.
To express the full-length and truncated GST-MGMT, GST-Ada, or GST-chimeric fusion proteins, E. coli ada^ogt^ colonies transformed with recombinant pGEX-3X plasmids, were grown overnight (10 ml LB broth, 100µg/ml ampicillin) and then diluted (inoculated 3ml of the culture into 30 ml LB media) and incubated at 37°C for 1 h in a shaking incubator. Fusion protein expression was induced by incubation of bacterial cultures with 0.1mM IPTG (25°C, 90 min). The bacterial cells were pelleted (3,000g, 10 min, 22°C), resuspended in 600 µl alkyltransferase buffer (0.05M Tris-HCl pH 8.0, 0.1mM EDTA, 5mM dithiothreitol, 5% glycerol), lysed on ice by mild sonication (3X-5 s bursts; 30 s pause between bursts) and repelleted (10,000g, 15 min, 4°C). The supernatant and pellet fractions, from induced and non-induced bacterial cultures (100 µg), were added to 1X SDS-reducing buffer (2X buffer= 0.125 M Tris-HCl pH 6.8, 20% glycerol, 2% SDS, 2% β-mercaptoethanol, 0.001% bromophenol blue) and heated at 95°C for 5 min. The debris from the pellet and supernatant fractions was pelleted by centrifugation at 10,000 g for 2 min. The supernatants from each fraction were analyzed for size on a 12.5% polyacrylamide-SDS gel using a 1X SDS-PAGE running buffer (20mM Tris-Base, 200mM glycine, 0.1% SDS, 5 h, 20 milliamps), along with a SDS-PAGE low molecular weight marker (BIO-RAD,
Bio-Rad Laboratories, Richmond, CA.) followed by Coomassie blue staining (0.05%) (described below). Bacterial supernatant fractions containing soluble GST-chimeric, GST-MGMT or GST-Ada fusion proteins were either directly assessed for repair of O⁶-MG in DNA or served as the starting material from which fusion or cleaved fusion proteins were isolated. For further purification, the bacterial supernatant (500 µg-1.0 mg total protein) containing fusion proteins was added to a glutathione Sepharose affinity column (equilibrated with 10 bed vol 1X PBS: 150mM NaCl, 16mM Na₂HPO₄, 4mM NaH₂PO₄, pH 7.3, 1% Triton X-100; Pharmacia LKB) and the eluent was discarded. The column was washed (3X-10 bed vol 1X PBS) and the glutathione-bound fusion protein was either eluted from the column with 2 ml glutathione buffer (5mM glutathione in 50mM Tris-HCl, pH 8.0) or cleaved from the column by addition of Factor Xa (1:100 enzyme:substrate molar ratio, 25°C, 3 h; Pierce, Rockford, IL) in a 500µl buffer containing 1% BSA, 5mM MES, 0.5M NaCl, pH 6.0. The fusion and hydrolyzed proteins (20µg) were fractionated by electrophoresis on a polyacrylamide-SDS gel along with a low molecular weight marker (BIO-RAD) followed by Coomassie blue (0.05%) or silver staining.
g. **Staining of SDS-Polyacrylamide Gels**

All full-length, mutant and chimeric MGMT and Ada fusion and hydrolyzed proteins, after separation on 12.5% polyacrylamide-SDS gels, were either Coomassie blue or silver stained. For Coomassie blue staining, the SDS-PAGE gels containing the GST-MGMT, GST-Ada, and GST-chimeric fusion proteins were soaked in 45% methanol, 10% acetic acid, 0.1% Coomassie blue R-250 (200 ml total volume) for 30 min on a rotator at 25°C. To destain, the gels were rinsed with several washes (3X- 30 min for each wash) of 20% methanol, 10% acetic acid. Prior to drying (described below), gels were soaked in 20% methanol, 3% glycerol overnight at 25°C.

For silver staining, the SDS-PAGE gels containing the factor-Xa cleaved control and mutant MGMT and Ada proteins were soaked in a 500 ml solution containing 10% acetic acid, 5% sulfosalicylic acid, 40% ethanol for 4 h on a rotator at 25°C. The gels were rinsed with 20% ethanol (4X- 20 min for each wash), and soaked in a 500 ml silver nitrate solution for 90 min. The silver nitrate solution was prepared by adding 25 ml 10% silver nitrate dropwise to a solution (475 ml) containing 20% ethanol, 0.15% NaOH, 1.0% NH₄OH, with constant stirring. The gels were washed with 20% ethanol (3X- 20 min for each wash) and soaked in a developing solution (prepared by mixing 0.5 ml 10% citric acid with 0.75 ml 37% formaldehyde in 500 ml dH₂O) for 15 min or until the
silver-stained protein bands appeared. The reaction was stopped by rinsing the gels in 1% acetic acid. The gels were washed in dH₂O (3X-20 min for each wash), wrapped in gel drying paper (Pharmacia, LKB) and dried under vacuum.

H. **Protein Quantitation**

Total protein content of bacterial sonicates containing fusion proteins, purified fusion proteins, and purified cleaved fusion protein preparations was determined by a Bradford protein assay program (Bio-rad assay; Bio-Rad Lab.) using as a standard bovine serum albumin (1-10µg) (Bradford, 1976).

Concentrations of non-purified control, truncated and chimeric MGMT and Ada fusion proteins were estimated as follows: non-purified fusion proteins (50µg total protein) were fractionated by electrophoresis on a 12.5% polyacrylamide-SDS gel and stained with Coomassie blue. The relative amount (µg) of each fusion protein was assessed by comparing the intensity of the Coomassie blue stained fusion protein band to that of the sum of all other protein bands.

Concentrations of purified control, truncated and chimeric MGMT and Ada fusion proteins (MGMT, MGMT-C28, MGMT-PCHRV-ADA, ADA, ADA-PCHRV-MGMT, and ADA-MGMTTAIL) were determined in two independent experiments. First, these purified fusion proteins (20 µg) were fractionated by
electrophoresis on a 12.5% polyacrylamide-SDS gel and stained with 0.05% Coomassie blue. The amount of each fusion protein was calculated by densitometric scanning (Tri Microscan System, Technology Res., Inc., Nashville, TN). The MGMT and Ada fusion protein content was assessed by comparing the optical density of the fusion protein band to that of a similarly fractionated bovine serum albumin protein band (1-25µg). Second, equivalent amounts (1.6 pmol) of MGMT, MGMT-C28, MGMT-PCHRV-ADA, ADA, ADA-PCHRV-MGMT, and ADA-MGMTTAIL, as calculated by densitometric analysis, were assessed in triplicate for the ability to completely repair varying amounts of an O\(^6\)-MG containing DNA substrate (1.0-2.2 pmol DNA substrate, 2 h, 37°C, as described below). Due to the stoichiometric nature of the repair reaction, the highest DNA concentration at which repair is complete is equal to the alkyltransferase concentration in the protein extract.

I. **Preparation and Labeling of an 18 bp O\(^6\)-MG-Containing DNA Substrate**

The synthetic 18-bp O\(^6\)-MG-containing oligodeoxynucleotide substrate was prepared by the hybridization of two complementary 18-mer and 16-mer oligodeoxynucleotide strands (Wu et al., 1987). The 18-mer (440 pmol) and 16-mer (400 pmol) were added to a 100 µl buffer (1mM EDTA, 10mM Tris-HCl pH 8.0, 33mM NaCl), denatured at 90°C for 10 min, and then quickly placed in a 70°C water
bath overnight (H₂O bath was immediately turned off to allow for hybridization). Following hybridization of the complementary strands, the 18-bp oligomer (4 pmol/µl), which contains two unpaired bases at one end, served as template for the Stoffel fragment of Taq DNA polymerase (fills in the 23' recessed end with thymidine 5'-[α-³²P]-triphosphate; Amersham, Arlington Heights, Il) (Figure 3). Twenty pmol of the 18-bp DNA substrate were incubated with 10 U2 Stoffel Taq DNA polymerase (Perkin Elmer/Cetus), 50μCi of [α-³²P]dTTP (specific activity 3000 Ci/mmol), 1.5mM MgCl₂, and 1X Stoffel Taq buffer (Perkin Elmer/Cetus) at 42°C for 1 h in a total volume of 20µl. Following DNA labeling, the 18-bp oligomer from this reaction was extracted (once with an equal volume phenol/24:1 chloroform-isooamy1 alcohol, and once with an equal volume 24:1 chloroform-isoamy1 alcohol), ethanol precipitated (0.1 vol 3M sodium acetate, 20µg tRNA, 2 vol ethanol, 1 h, -80°C), pelleted (12,000 g, 30 min) and dried under vacuum. The ³²P-end-labeled 18-bp oligodeoxynucleotide substrate was resuspended in 100µl dH₂O (final concentration assumed to be 0.2 pmol/µl) and stored at 4°C.
Figure 3. Specific sequence of the oligodeoxynucleotide probe. The $^{32}$P-end-labeled 18 bp oligonucleotide substrate contains a single methyl lesion at the O$_6$-position of guanine within a PvuII restriction site. Vertical lines, position of cleavage of the restriction enzymes HaeIII or PvuII. Horizontal line, defines the PvuII restriction site (Wu et al., 1987).
J. Assay for Repair of O⁶-MG in DNA

Repair of O⁶-MG containing DNA by four MGMT oligopeptides and bacterial sonicates containing control, truncated or chimeric MGMT and Ada fusion proteins was assessed in triplicate by a restriction endonuclease inhibition assay (Wu et al., 1987; Futscher et al., 1989). MGMT oligopeptides 1-4 (25 µg) and ada⁻ogt⁻ cell extracts containing the control or mutant Ada or MGMT fusion proteins (0-200 µg) were reacted separately with 0.2 pmol of a ³²P-end labeled 18 bp O⁶-MG containing DNA substrate at 37°C for 2 h in 150µl alkyltransferase buffer. As described, the 18-bp oligonucleotide substrate contains a single methyl lesion at the O⁶-position of guanine within a PvuII site (Figure 3) (Wu et al., 1987). After incubation, the DNA from this reaction was extracted, ethanol precipitated, pelleted (12,000xg, 30 min) and dried under vacuum. The DNA was resuspended in 17µl dH₂O and 2µl 10X react 6 buffer (BRL) and then incubated with PvuII (10U, 37°C, 1 h). Three unreacted control samples were incubated with either no restriction endonuclease, PvuII, or HaeIII (10U, 37°C, 1 h). All digestion reactions were terminated by addition of 9µl of 95% formamide dye (95% formamide, 1mM EDTA, 0.1% Bromophenol blue, 0.1% Xylene Cyanol). Following denaturation at 95°C, the DNA sample was quickly cooled on ice and fractionated by electrophoresis (10 µl aliquots) on a 20% denaturing polyacrylamide gel (26
milliamps, 1 h). The amount of restriction enzyme-cleaved DNA substrate was visualized through direct gel autoradiography (18 h, -80°C), quantitated by analysis on a Betascope 630 (Betagen, Inc.), and compared to the amount of DNA uncleaved in each sample. Repair activity was expressed as percent PvuII-mediated cleavage of the protein-reacted DNA relative to HaeIII-mediated cleavage of the same DNA substrate.

The rate of repair of the 18-bp O\(^{6}\)-MG-containing DNA substrate by MGMT, MGMT-C28, and Ada purified fusion proteins was measured in triplicate at 4°C and 37°C using the restriction endonuclease inhibition assay as described above. For analysis at 4°C, the \(^{32}\)P-end-labeled 18-bp DNA substrate (0.2 pmol) was incubated for various times (0-400 min) with Ada and MGMT purified fusion proteins (1.6 pmol) in a 150\(\mu\)l total reaction volume. For analysis at 37°C, purified MGMT fusion proteins (1.6 pmol) were reacted with the DNA substrate (0.02 pmol) for 0-30 min in a 300\(\mu\)l total reaction volume.

K. **Analysis of O\(^{6}\)-BG Repair Activity**

The ability of MGMT oligopeptides and bacterial sonicates containing MGMT control and truncated fusion proteins to repair 8-[\(^{3}\)H]-O\(^{6}\)-benzylguanine was determined in triplicate by incubating 11.5 pmol of 8-[\(^{3}\)H]-O\(^{6}\)-benzylguanine
with an estimated 100-fold molar excess of Peptides 1-4, MGMT-C35, MGMT-N18, MGMT-N78, MGMT, or Ada fusion proteins in 20 µl alkyltransferase buffer for 2 h at 37°C. 8-[^3H]-O^6^-benzylguanine was prepared by Amersham Corporation and was purified by M.E. Dolan (1991). A control incubation without added protein was carried out for the same time in the same buffer. After incubation, the reactions were terminated by addition of 500 µl of the reaction buffer containing unlabeled 200 µM guanine (Sigma, St. Louis, MO) and 200 µM benzylguanine (provided by R.C. Moschel). Measurements of 8-[^3H]guanine formation from 8-[^3H]-O^6^-benzylguanine for each sample were determined by reverse phase HPLC separation (Rainin Lichrosorb RP18 column) of the compounds. The samples (520 µl) were eluted with 50% methanol/0.05M ammonium formate pH 4.5 at 35°C with a flow rate of 1 mL/min. Authentic guanine (retention time= 4 min) and O^6^-benzylguanine (retention time= 10 min) were detected using a Waters UV detector at a wavelength of 280 nm. Radioactivity corresponding to these peaks was monitored by use of a radioactivity flow detector (Radiomatic, Inc., Tampa, Fl.).

The rate of O^6^-BG repair by full-length, truncated, and chimeric MGMT and Ada fusion proteins was measured in triplicate by incubating O^6^-BG (16 pmol) with Ada, MGMT, MGMT-C28, ADA-PCHRVMGMT, MGMT-PCHRVA-ADA, and ADA-MGMTTAIL (1.6 pmol) purified fusion proteins and ADA-LELS-MGMT, MGMT-GLHE-ADA, MGMT-ADA1-MGMT, MGMT-ADA2-MGMT, MGMT-ADA3-MGMT, and
MGMT-ADA5-MGMT (50µg) non-purified fusion proteins in 300µl alkyltransferase buffer for 0-30 min at 37°C. Following O\(^6\)-BG pre-exposure, 0.02 pmol of \(^{32}\)P-end labeled O\(^6\)-MG containing DNA were added, in the presence of O\(^6\)-BG, and the reaction was further incubated for 2 h at 37°C. BG-mediated inhibition of O\(^6\)-MG repair was indirectly monitored by the restriction endonuclease inhibition assay (described above). O\(^6\)-BG-mediated inactivation was expressed as percent inhibition of PvuII-mediated cleavage of the protein-reacted DNA relative to non-PvuII digested DNA.

L. **Assay for Repair of O\(^4\)-MT in DNA**

The ability of MGMT, Ada, and chimeric fusion proteins to repair O\(^4\)-methyl\(^{3}\)H]thymine in poly(dA)-poly(dT) was determined in triplicate by incubating N-\(^{3}\)H]methyl-N-nitrosourea-exposed poly(dA)-poly(dT) substrate (approximately 0.5 pmol O\(^4\)-methyl\(^{3}\)H]thymine) with a 100-fold molar excess of purified MGMT, Ada, MGMT-PCHRV-ADA, or ADA-PCHRV-MGMT fusion proteins in 50µl alkyltransferase buffer for 2 h at 37°C. A control incubation without added protein was carried out for the same time in the same buffer. \(^{3}\)H]methylated poly(dT)/poly(dA) substrate was prepared by the reaction of 25 units poly(dT) with 0.1mCi N-\(^{3}\)H]methyl-N-nitrosourea (Amersham Corp.) in 100µl 0.05M Tris, pH 8.0 at 37°C for 1 h. The alkylated poly(dT) was extracted (once
with an equal volume of phenol/24:1 chloroform-isoamyl alcohol, and once with an equal volume 24:1 chloroform-isoamyl alcohol), ethanol precipitated (0.1 vol 3M sodium acetate, 2 vol ethanol, 1 h, -80°C), pelleted (16,000g, 30 min, 4°C) and dried under vacuum. The poly(dT) pellet was resuspended in 0.05M Tris, pH 8.0 and annealed to 25 units poly(dA) in 500µl of the same buffer at 25°C for 2 h. Each sample described above was cooled to 0°C and incubated with deoxyribonuclease I (660u/mL) (Sigma) in 500µl 0.01M magnesium acetate pH 6.5 buffer for 1 h at 37°C. E. coli alkaline phosphatase (8u/mL) (Sigma) and snake venom phosphodiesterase (0.2u/mL) (Pharmacia, LKB) were subsequently added (pH of the magnesium acetate buffer was adjusted to 8.0) for 18 h at 37°C. Proteins were precipitated (95°C, 10 min) and pelleted (16,000g, 1 h), and the remaining supernatant was analyzed for O4'-methylthymine, 3-methylthymine, and O2'-methylthymine by reverse phase HPLC separation (Rainin Lichrosorb RP18 column) of the compounds. The samples (250 µl total volume) were eluted with 20% methanol/0.05M ammonium formate pH 4.5 at 35°C with a flow rate of 1mL/min and the columns were washed with 100% methanol for 30 min. O4'-methylthymine (retention time= 18 min), 3-methylthymine (retention time= 12 min), and O2'-methylthymine (retention time= 7.5 min) (Chemsyn Science Lab., Lenexa, KS) were detected using a Waters UV detector at a wavelength of 254nm. Radioactivity from sample fractions (1 fraction/min collected) corresponding to these
peaks was monitored through use of a scintillation counter. 
O⁴-MT repair was determined by comparing O⁴-methyl[³H] thymine content in poly(dA)-poly(dT) exposed to full-length or chimeric MGMT and Ada proteins to that in control poly(dA)-poly(dT) substrate.
CHAPTER IV
RESULTS

A. Construction of Full-Length, Truncated, and Chimeric MGMT and Ada Fusion Proteins

1. Construction of GST-MGMT, GST-Ada, and GST-chimeric bacterial expression vectors

All full-length and truncated human MGMT and E. coli ada cDNAs were synthesized by PCR and all MGMT-ada, ada-MGMT, and MGMT-ada-MGMT chimeric cDNAs were generated using the PCR overlap extension technique (Ho et al., 1989). These products were generated using conditions (high template concentration, low cycle number) demonstrated to minimize Taq polymerase-induced mutation (Ho et al., 1989). All products were of the predicted size as assessed by agarose gel electrophoresis next to molecular weight markers (representative examples are shown in figure 4) (Demple et al., 1985; Tano et al., 1990). Each gel-purified, PCR-generated cDNA, after sequential EcoRI and BamHI digestion, was subcloned into the pGEX-3X bacterial expression vector which in turn directs the synthesis of glutathione S-transferase (GST)-MGMT, GST-ADA, and GST-chimeric fusion proteins in E. coli under the control of the
Figure 4. PCR amplification of control and mutant MGMT and ada cDNAs. MGMT (645 bp), ADA-PCHRV-MGMT (630 bp), ADA (553 bp), MGMT-PCHRV-ADA (553 bp), AND MGMT-C28 (549 bp) cDNAs (Lanes 1-5, respectively) were synthesized by PCR and subsequently analyzed by electrophoresis on an ethidium bromide-containing 1.0% agarose gel and photographed. Sizes of a 123-bp ladder are also indicated.
IPTG-inducible tac promoter. Sequencing (approximately 50-70 nt) across the fusion sites of all MGMT-ada and ada-MGMT chimeric cDNAs, and the junction of either the 3' or 5' end of the MGMT cDNAs and the bacterial expression vector, confirmed that in each case the desired cDNA fusion or deletion was accomplished. Sequencing of the fusion sites of the five MGMT-ada-MGMT chimeric cDNAs confirmed that the desired ada nt substitutions were accomplished. One exception, however, was the MGMT-ada4-MGMT construct which, for those cDNAs analyzed, was found to be the wildtype MGMT cDNA sequence. All subsequent studies, therefore, excluded the analysis of MGMT-ADA4-MGMT.

2. Expression and isolation of GST-MGMT, GST-Ada, and GST-chimeric fusion proteins

To produce soluble full-length, truncated, or chimeric MGMT and Ada fusion proteins, ada-ogt- cells transformed with recombinant pGEX-MGMT, pGEX-ada, or pGEX-chimeric cDNA constructs were incubated with IPTG at room temperature for 90 min. Supernatant and pellet fractions (100µg) derived from ada-ogt- whole cell sonicates containing these fusion proteins were analyzed on a series of polyacrylamide-SDS gels to demonstrate that all proteins were stable, and of the predicted size encoded by the expression vectors. Diagrams of full-length, truncated, and chimeric MGMT and Ada fusion proteins, in addition to the MGMT oligopeptides, are shown in
Figure 5. Diagrams of the full-length, truncated, and chimeric MGMT and Ada fusion proteins and MGMT oligopeptides. All full-length, truncated, or chimeric MGMT (hatched box) and Ada (open box) constructs were expressed as GST-fusion proteins. The thick and thin black boxes indicate the MGMT 28 aa carboxyl-terminal tail and the PCHRV acceptor site, respectively. The names of each truncated MGMT protein designate the aa deletion made at either the amino- or carboxyl-terminus. The names of MGMT/Ada and Ada/MGMT chimeric proteins indicate the aa sequences joining the two proteins. The MGMT-ADA-MGMT chimeric proteins each contain approximately 10-20 Ada aa in place of MGMT aa. The small Ada aa substitutions for the MGMT-ADA-MGMT proteins span from the MGMT amino-terminal GLHE region to the PCHRV acceptor site. All MGMT oligopeptides contain the conserved PCHRV acceptor site.
Figure 5. Figure 6 represents an SDS-PAGE analysis of supernatant fractions derived from ada-ogt- whole cell sonicates containing either MGMT (48 kDa), MGMT-C65 (41 kDa, faint in this preparation), MGMT-C10 (47 kDa), MGMT-C28 (45 kDa), or Ada (45 kDa) (lanes 3-7, respectively). Arrows indicate the fusion protein of the predicted size encoded by the expression vectors. These proteins were expressed only in bacteria pre-exposed to IPTG and were not present in non-transformed bacteria (Figure 6, lane 2) or in HT-29 cell sonicates (Figure 6, lane 1). Figure 7 represents a similar SDS-PAGE analysis of pellet and supernatant fractions derived from IPTG-induced bacterial cultures containing either MGMT-C35 (44 kDa) (lanes 5, 6), MGMT-N18 (46 kDa) (lanes 9, 10), or MGMT-N78 (39 kDa, faint in this preparation) (lanes 13, 14). All fusion proteins, as indicated by the arrows, were of the predicted size and all soluble proteins were readily detectable in supernatant fractions. These proteins, however, were not present in supernatant and pellet fractions from either non-transformed, sonicated ada-ogt- cells (Figure 7, lanes 1, 2) or from non-IPTG induced bacterial cells transformed with MGMT cDNA constructs encoding either MGMT-C35 (lanes 3, 4), MGMT-N18 (lanes 7, 8), or MGMT-N78 (lanes 11, 12). SDS-PAGE analysis of supernatant and pellet fractions derived from ada-ogt- whole cell sonicates containing either ADA-PCHRV-MGMT (48 kDa) (Figure 8, lanes 1, 2), MGMT-PCHRV-ADA (45 kDa) (lanes 3, 4), ADA-MGTTAIL (48
Figure 6. SDS-PAGE analysis of ada'ogt' whole cell sonicates containing either GST-MGMT or GST-Ada fusion proteins. Alkyltransferase deficient whole cell sonicates (100 µg total protein/lane) containing IPTG-induced MGMT(48kDa), MGMT-C65 (41kDa), MGMT-C10 (47kDa), MGMT-C28 (45 kDa), or Ada (45kDa) fusion proteins (Lanes 3-7, respectively) were fractionated by electrophoresis through a 12.5% polyacrylamide-SDS gel followed by Coomassie blue staining (0.05%). Arrows indicate the fusion protein of the predicted size. Positions and sizes of a Bio-Rad SDS-PAGE low molecular weight standard are indicated. These fusion proteins were not present in HT-29 colon carcinoma cell sonicates (lane 1) or in non-transformed ada'ogt' cell sonicates (lane 2).
Figure 7. SDS-PAGE analysis of ada-ogt- whole cell sonicates containing either MGMT-C35, MGMT-N18, or MGMT-N78 fusion proteins. Pellet (P) and supernatant (S) fractions (100 µg total protein/lane) derived from IPTG-induced bacterial cultures containing either MGMT-C35 (44 kDa) (lanes 5, 6), MGMT-N18 (46 kDa) (lanes 9, 10), or MGMT-N78 (39 kDa) (lanes 13, 14) fusion proteins were fractionated by electrophoresis through a 12.5% polyacrylamide SDS-gel followed by Coomassie blue staining (0.05%). Arrows indicate the fusion protein of the predicted size. These fusion proteins were not present in supernatant and pellet fractions from either non-transformed, sonicated ada-ogt- cells (lanes 1, 2) or from non-IPTG induced bacterial cells transformed with MGMT cDNA constructs encoding either MGMT-C35 (lanes 3, 4), MGMT-N18 (lanes 7, 8), or MGMT-N78 (lanes 11, 12).
Figure 8. SDS-PAGE analysis of ada\textsuperscript{-}ogt\textsuperscript{-} whole cell sonicates containing either ADA-PCHRV-MGMT, MGMT-PCHRV-ADA, or ADA-MGMTTAIL fusion proteins. Supernatant (s) and pellet (p) fractions (100\mu g total protein/lane) derived from IPTG-induced bacterial cultures containing either ADA-PCHRV-MGMT (48 kDa) (lanes 1, 2), MGMT-PCHRV-ADA (45 kDa) (lanes 3, 4), or ADA-MGMTTAIL (48 kDa) (lanes 5, 6) fusion proteins were fractionated by electrophoresis through a 12.5% polyacrylamide SDS-gel followed by Coomassie blue staining (0.05%). Arrows indicate the fusion protein of the predicted size. These fusion proteins were not present in non-transformed ada\textsuperscript{-}ogt\textsuperscript{-} cell sonicates (lane 7).
48 kDa) (lanes 5, 6), MGMT-ADA1-MGMT (48 kDa) (Figure 9, lanes 2, 3), MGMT-ADA2-MGMT (48 kDa) (lanes 4, 5), MGMT-ADA3-MGMT (48 kDa) (lanes 6, 7), MGMT-ADA5-MGMT (48 kDa) (lanes 8, 9), ADA-LELS-MGMT (48 kDa) (lanes 10, 11), or MGMT-GLHE-ADA (45 kDa) (lanes 12, 13) yielded similar results (i.e. all fusion proteins were soluble and of the predicted, and were not present in nontransformed bacteria) (Figure 8, lane 7; Figure 9, lane 1).

Bacterial supernatant fractions containing soluble GST-chimeric, GST-MGMT, or GST-Ada fusion proteins were either directly assessed for repair of O6-MG-containing DNA (described below), or were further purified by affinity chromatography on immobilized glutathione. The purified fusion and factor Xa-cleaved fusion proteins were analyzed for size and purity on a polyacrylamide-SDS gel followed by Coomassie blue or silver staining. SDS-PAGE analysis of purified MGMT, ADA-MGMTTAIL, Ada, MGMT-C28 (Figure 10, lanes 1-4, respectively), ADA-PCHRV-MGMT, MGMT-PCHRV-ADA (Figure 11, lanes 1, 2, respectively), MGMT-ADA1-MGMT, MGMT-ADA2-MGMT, MGMT-ADA3-MGMT, and MGMT-ADA5-MGMT (Figure 12, lanes 1-4, respectively) GST-fusion proteins (20 µg) demonstrated that these proteins were the predominant peptide in each preparation. These GST-fusion proteins represented, by densitometric scan of the gel, approximately 80% of the total protein. This represents a greater than 10-fold purification relative to the non-fractionated sonicates containing the
Figure 9. SDS-PAGE analysis of ada-o gt whole cell sonicates containing MGMT and Ada chimeric fusion proteins. Supernatant (s) and pellet (p) fractions (100µg total protein/lane) derived from IPTG-induced bacterial cultures containing either MGMT-ADA1-MGMT (48 kDa) (lanes 2, 3), MGMT-ADA2-MGMT (48 kDa) (lanes 4, 5), MGMT-ADA3-MGMT (48 kDa) (lanes 6, 7), MGMT-ADA5-MGMT (48 kDa) (lanes 8, 9), ADA-LELS-MGMT (48 kDa) (lanes 10, 11), or MGMT-GLHE-ADA (45 kDa) (lanes 12, 13) fusion proteins were fractionated by electrophoresis through a 12.5% polyacrylamide SDS-gel followed by Coomassie blue staining (0.05%). Arrows indicate the fusion protein of the predicted size. Positions and sizes of a Bio-Rad SDS-PAGE low molecular weight standard are also indicated. These fusion proteins were not present in non-transformed ada-o gt cell sonicates (lane 1).
Figure 10. SDS-PAGE analysis of GST-affinity purified MGMT and Ada GST-fusion proteins. Purified MGMT (48 kDa), ADA-MGMTTAIL (48 kDa), ADA (45 kDa), and MGMT-C28 (45 kDa) (lanes 1-4, respectively) GST-fusion proteins (20µg/lane) were fractionated by electrophoresis through a 12.5% polyacrylamide SDS-gel followed by Coomassie blue staining (0.05%). Arrows indicate the purified fusion protein of the predicted size (positions and sizes of a Bio-Rad SDS-PAGE low molecular weight standard are also indicated).
Figure 11. SDS-PAGE analysis of GST-affinity purified ADA-PCHRV-MGMT and MGMT-PCHRV-ADA GST-fusion proteins. Purified ADA-PCHRV-MGMT (48 kDa) and MGMT-PCHRV-ADA (45 kDa) (lanes 1, 2, respectively) fusion proteins (20µg/lane) were fractionated by electrophoresis through a 12.5% polyacrylamide SDS-gel followed by Coomassie blue staining (0.05%). Arrows indicate the predominant fusion protein of the predicted size.
fusion proteins. Additionally, the control and mutant MGMT and Ada fusion proteins were not present in affinity purified nontransformed ada-ogt- whole cell sonicates (data not shown). The presence of purified, silver-stained cleaved fusion proteins (13-22 kDa) could not be demonstrated as these proteins presumably co-migrated on an SDS-polyacrylamide gel with various endogenously produced E. coli proteins. Therefore, purified GST-fusion proteins (or bacterial supernatant fractions containing GST-fusion proteins) were used in subsequent DNA repair studies.

B. The Role of the 28 Amino Acid Carboxyl-Terminal Tail in MGMT Activity, Temperature Sensitivity, and Substrate Specificity

1. Repair of O\textsuperscript{6}-MG in DNA by control MGMT and carboxyl-terminal MGMT deletion proteins

Because the MGMT 28 aa carboxyl-terminal tail is one of the most notable structural differences among mammalian and bacterial alkyltransferases, this tail may play a unique role in MGMT function. To determine the significance of the carboxyl-terminal 28 aa sequence in MGMT repair activity, a restriction endonuclease inhibition assay was used (Wu et al., 1987; Futscher et al., 1989). This assay measures the extent to which human MGMT, E. coli Ada, and carboxyl-terminal MGMT deletion proteins repair O\textsuperscript{6}-MG lesions within a PvuII site (CAGCTG\textsuperscript{mme}) of a $^{32}\text{P}$-end labeled, 18-bp DNA
Figure 12. SDS-PAGE analysis of GST-affinity purified MGMT-ADA-MGMT GST-fusion proteins. Purified MGMT-ADA1-MGMT (48 kDa), MGMT-ADA2-MGMT (48 kDa), MGMT-ADA3-MGMT (48 kDa), and MGMT-ADA5-MGMT (48 kDa) (lanes 1-4, respectively) fusion proteins (20µg/lane) were fractionated by electrophoresis through a 12.5% polyacrylamide SDS-gel followed by Coomassie blue staining (0.05%). Arrows indicate the purified fusion protein of the predicted size (positions and sizes of a Bio-Rad SDS-PAGE low molecular weight standard are also indicated).
substrate (Wu et al., 1987). Preincubation of the damaged DNA substrate with repair proficient protein extracts allows for removal of the methyl group from the O6-position of guanine. Subsequent incubation with PvuII allows cleavage of the DNA to generate a labeled 8-bp fragment and an unlabeled 10-bp fragment. The 8-bp and 10-bp DNA are separated from the unrepaired and uncleaved 18-bp DNA containing O6-MG by electrophoresis on a 20% denaturing polyacrylamide gel. The relative proportion of radiolabeled 8-nt to 18-nt DNA, after direct gel autoradiography, is quantitatively measured using a Betascope 630 (Betagen Inc.). The amount of alkyltransferase activity in each protein extract is proportional to the ratio of 8-nt to 18-nt+8-nt DNA.

Figure 13 represents an autoradiograph from an alkyltransferase assay in which the 18-bp DNA substrate was cleaved with PvuII following reaction with sonicates (50µg total protein) of ada'ogt' cells transformed with MGMT or Ada cDNA constructs, encoding MGMT, MGMT-C65, MGMT-C10, MGMT-C28, or Ada fusion proteins (lanes 6-10, respectively). In this, and all alkyltransferase assays to be presented, the labeled DNA fragment, prior to protein incubation and PvuII cleavage, migrated as a single 18-nt labeled fragment (Figure 13, lane 1). This DNA was completely resistant to PvuII digestion without prior exposure to alkyltransferase-containing protein sonicates (Figure 13, Lane 2) although the DNA was cleaved to
Figure 13. Repair of O\textsuperscript{6}-MG in DNA by control and truncated MGMT and Ada fusion proteins. Sonicates (50µg total protein) of ada\textsuperscript{-}ogt\textsuperscript{-} cells transformed with MGMT or Ada cDNA constructs encoding MGMT, MGMT-C65, MGMT-C10, MGMT-C28, or Ada fusion proteins (lanes 6-10, respectively) were reacted with a \textsuperscript{32}P\textsuperscript{-}end labeled 18 bp DNA substrate (2 h, 37°C) which contains an O\textsuperscript{6}-MG lesion blocking a PvuII restriction enzyme recognition site (see figure 3). Following phenol/chloroform extraction and ethanol precipitation, the DNA was digested with PvuII (10U, 1h, 37°C). Labeled DNA fragments were analyzed for size on a 20% polyacrylamide gel (26 mA, 1 h). The 8 bp fragment was generated by PvuII cleavage of the repaired 18 bp fragment following incubation with MGMT or Ada fusion proteins. The first three control lanes are 1; unreacted, undigested 18 bp DNA, 2; unreacted PvuII digested 18 bp DNA, 3; unreacted HaeIII digested 18 bp DNA. Lanes 4 and 5 represent the PvuII digestion products of the 18bp DNA substrate after incubation with either non-transformed bacterial sonicates (50µg) or HT-29 colon carcinoma cell sonicates (50µg), respectively.
a 12-bp fragment by HaeIII which is not inhibited by the O\textsuperscript{6}-MG lesion (Figure 13, Lane 3). The same labeled DNA, if pre-exposed to sonicates of MGMT-expressing human HT-29 colon carcinoma cells (Figure 13, Lane 5), sonicates of bacteria transformed with either MGMT cDNA constructs encoding MGMT (Figure 13, lane 6), MGMT-C10 (Figure 13, lane 8), or MGMT-C28 (Figure 13, lane 9) or ada DNA constructs encoding Ada (Figure 13, lane 10), was cleaved to a labeled 8-nt fragment by PvuII. The presence of the 8-nt fragment is indicative of alkyltransferase activity in these protein extracts. Alkyltransferase activity, however, was absent in non-transformed ada\textsuperscript{ogt}\textsuperscript{cell} sonicates (Figure 13, Lane 4) and in sonicates containing MGMT-C65 which lacks sequences encoding for the PCHRV acceptor site (Figure 13, lane 7). These results demonstrate that up to 28 aa at the MGMT carboxy-terminus can be deleted without loss of MGMT activity. Although GST-fusion proteins were used, fusion of the GST peptide onto the Ada and MGMT constructs did not appear to affect alkyltransferase stability or repair activity. This observation is confirmed by the fact that the full-length GST-MGMT protein appeared to exhibit DNA repair activity comparable to that of endogenous MGMT in HT-29 colon carcinoma cell extracts. Additionally, based on the Ada crystal structure, the GST peptide appears to be fused to a proposed amino-terminal structural domain which is independent from the protein's active site. This observation
suggests that the GST peptide would not directly alter protein activity.

2. Quantitation of active MGMT and MGMT-C28 fusion proteins

Although the carboxyl-terminal tail appears to be dispensable for MGMT activity, the possibility exists that deletion of the tail affects the rate of MGMT-mediated repair of O\(^6\)-MG in DNA. To assess this possibility, amounts of active purified MGMT and MGMT-C28 fusion proteins were first determined using an alkyltransferase activity assay in which 1.6 pmol of fusion protein were reacted with an increasing pmol amount (1.0-2.0) of a \(^{32}\)P-end labeled 18bp DNA substrate. At a molar excess of MGMT and MGMT-C28, MGMT activity for both proteins was 99\% \pm 3.0 (Figure 14). In this and all other figures, repair activity was expressed as percent \(Pvu\text{II}\)-mediated cleavage of protein-reacted DNA relative to percent \(Hae\text{III}\)-mediated cleavage (95\% \pm 5.0) of the same DNA substrate. The extent of DNA substrate repair decreased, however, in reactions wherein the molar DNA substrate concentration exceeded the molar concentration of MGMT and MGMT-C28. The highest molar concentration of DNA substrate that was 100\% repaired is equivalent to the molar amount of active fusion protein used for that given reaction. The quantitation of MGMT and MGMT-C28 fusion proteins by this functional analysis (1.3 pmol for both proteins) was in close
Figure 14. Quantitation of control and mutant MGMT fusion proteins. MGMT (closed symbols) and MGMT-C28 (open symbols) fusion proteins (1.6 pmol each) were assayed for MGMT repair activity by reaction in triplicate with an increasing picomole amount (1.0-2.0) of a $^{32}$P-end labeled O$^6$-MG-containing DNA fragment for 2h at 37°C. Repair activity was expressed as percent PvuII-mediated cleavage of DNA substrate relative to HaeIII-mediated cleavage of undamaged DNA.
agreement with that derived by densitometric scanning of the Coomassie blue stained gel containing MGMT and MGMT-C28 fusion proteins (1.6 pmol). These results indicate that MGMT-C28 exhibited similar activity to MGMT, and given a molar excess of protein, both MGMT and MGMT-C28 will completely repair O\textsuperscript{6}-MG in DNA.

3. Effect of 28 amino acid carboxyl-terminal tail deletion on the rate of MGMT-mediated repair of O\textsuperscript{6}-MG-containing DNA at 4°C

The repair of O\textsuperscript{6}-MG in DNA by MGMT and MGMT-C28 was determined using the restriction endonuclease inhibition assay. DNA repair was assessed following varying times of incubation of O\textsuperscript{6}-MG containing DNA substrate with a 100-fold molar excess of full-length or mutant MGMT fusion proteins at different temperatures. At 37°C, 25°C, and 15°C, repair by both proteins was very rapid and was complete by the first time point (30 s, data not shown) whereas reducing the reaction temperature to a randomly chosen 4°C slowed the reaction rate significantly. Figure 15 illustrates the results of an alkyltransferase assay in which a 100-fold molar excess (1.6 pmol) of MGMT, MGMT-C28, or Ada fusion proteins were incubated for varying times with the 18bp DNA substrate at 4°C. The Ada and MGMT fusion proteins completed DNA repair within 5 and 30 min, respectively, following DNA substrate addition. The MGMT-C28 fusion protein, however,
Figure 15. Effect of 28 aa carboxyl-terminal tail deletion on the rate of MGMT-mediated repair of O\textsuperscript{6}-MG-containing DNA at 4°C. MGMT (closed triangles), MGMT-C28 (open triangles), and Ada (open circles) fusion proteins (1.6 pmol) were assayed for alkyltransferase repair activity by reaction in triplicate with 0.2 pmol of a \textsuperscript{32}P-end labeled 18bp DNA substrate for 0 to 400 min at 4°C.
repaired only 25% of the DNA substrate following even a 6 h incubation. To determine whether MGMT-C28 was permanently inactivated at lower temperatures, a 100-fold molar excess of MGMT-C28 was first reacted with the DNA substrate for 1 h at 4°C and then for an additional hour at 37°C. Results in Figure 16 show that once MGMT-C28 was incubated for 1 h at 37°C, following a 1 h 4°C incubation (Figure 16, dotted line), the amount of repair was comparable to that mediated by MGMT after 1 h at 37°C alone (Figure 16, solid line). These results indicate that deletion of the 28 aa carboxyl-terminal tail reversibly inhibits the ability of the protein to repair O⁶-MG in DNA at 4°C, suggesting that the carboxyl-terminal tail affects MGMT activity at lower temperatures.

4. Effect of 28 amino acid carboxyl-terminal tail deletion on the rate of MGMT-mediated repair of O⁶-MG-containing DNA at 37°C

Because of the temperature sensitive nature of the MGMT-C28 fusion protein, the rates of repair of O⁶-MG in DNA by MGMT and MGMT-C28 were assessed using a 37°C incubation and a 10-fold decreased molar concentration of protein:DNA substrate, which in turn slowed the reaction and allowed accurate assessment of the time course of repair. Using these conditions, the MGMT and MGMT-C28 fusion proteins had equivalent rates of repair of O⁶-MG in DNA. Both proteins repaired approximately 25% of the substrate within 5 min and
Figure 16. Analysis of the temperature sensitivity of the MGMT-C28 mutant fusion protein. MGMT (1.6 pmol) (solid line) was assayed for MGMT activity by reaction in triplicate with 0.02 pmol of a $^{32}$P-end labeled 18bp DNA substrate for 0, 60, and 120 min at 37°C. MGMT-C28 (1.6 pmol) (dotted line) was assayed for MGMT activity by reaction with 0.2 pmol of a $^{32}$P-end labeled 18bp DNA substrate for 0 min at 4°C, 60 min at 4°C and then an additional 60 min at 37°C, and for 120 min at 4°C.
Figure 17. Effect of 28 aa carboxyl-terminal tail deletion on the rate of MGMT-mediated repair of $\text{O}^6$-MG-containing DNA at 37°C. MGMT (closed symbols) and MGMT-C28 (open symbols) fusion proteins (1.6 pmol) were assayed for MGMT repair activity by reaction in triplicate with 0.002 pmol of a $^{32}$P-end labeled 18bp DNA substrate for 0 to 30 min at 37°C.
100% of the substrate within 30 min (Figure 17). These results indicate that the rate of MGMT-mediated repair of O\textsuperscript{6}MG in DNA at 37°C is not affected by deletion of the carboxyl-terminal tail.

5. Effect of 28 amino acid carboxyl-terminal tail deletion on the rate of MGMT-mediated repair of O\textsuperscript{6}-BG

While the tail sequence appears to be absolutely dispensable for MGMT-mediated repair of O\textsuperscript{6}-MG in DNA, the possibility exists that divergent regions such as the carboxyl-terminal tail may play a role in alkyltransferase substrate specificity. Specifically, this carboxyl-terminal tail, which is lacking in Ada, may uniquely contribute to the ability of MGMT to repair larger, bulkier lesions such as O\textsuperscript{6}-BG. To assess the role the tail plays in O\textsuperscript{6}-BG repair, MGMT, MGMT-C28, and Ada fusion proteins, and HT-29 cell sonicates containing endogenous MGMT protein, were indirectly analyzed for the rates of repair of O\textsuperscript{6}-BG. These proteins (1.6 pmol) were incubated for varying times with a 10-fold molar excess of O\textsuperscript{6}-BG followed by the addition of the 18-bp DNA substrate previously described. The ability of the various proteins to repair O\textsuperscript{6}-BG was measured as inhibition of the repair of O\textsuperscript{6}-MG in DNA by BG pre-incubation using the restriction endonuclease inhibition assay. As described previously (Dolan et al., 1991), Ada, which served as a negative control, was unaffected by O\textsuperscript{6}-BG pre-exposure (Figure 18,
Figure 18. Effect of 28 aa carboxyl-terminal tail deletion on the rate of MGMT-mediated repair of O6-BG. MGMT (closed triangle), MGMT-C28 (open triangle), and Ada (open circle) fusion proteins (1.6 pmol) and an equimolar amount of endogenous MGMT (closed circles) in HT-29 colon carcinoma cell sonicates were reacted in triplicate with 16 pmol of O6-BG for 0 to 10 min at 37°C. These proteins were subsequently incubated with the 18bp DNA substrate for 2 h. O6-BG mediated inhibition of O6-MG repair was monitored by the restriction endonuclease inhibition assay as described in figure 13. O6-BG-mediated inactivation was expressed as percent inhibition of PvuII-mediated cleavage of the protein-reacted DNA relative to non-PvuII-digested DNA.
0-12 min). In contrast, MGMT-expressing HT-29 cell sonicates (10µg), serving as a positive control, were partially inactivated (as measured by inhibition of PvuII-mediated cleavage of the protein-reacted DNA, relative to non-PvuII digested DNA) within seconds of O⁶-BG exposure (Figure 18, 0 time point), and were completely inactivated within 2 min. The time course of O⁶-BG inactivation of the MGMT fusion protein was similar to that of the human MGMT in cell extracts with both proteins being completely inactivated within 2 min. The rate of O⁶-BG-mediated inactivation of MGMT-C28 was, however, different from that of the MGMT fusion protein. Although both proteins were completely inactivated by O⁶-BG within 10 min, the rate of inactivation of MGMT-C28 compared to MGMT was decreased at least 5-fold (Figure 18), as measured by the O⁶-BG exposure necessary to cause 50% inactivation (2 min for MGMT-C28 and <30 s for MGMT). These results indicate that deletion of the MGMT 28 aa carboxyl-terminal tail decreases the rate of MGMT-mediated repair of O⁶-BG, suggesting that the carboxyl-terminal tail plays an indirect role in MGMT-mediated O⁶-BG repair activity.

6. Repair of O⁶-BG by ADA-MGMTTAIL fusion protein

The exact mechanism by which the carboxyl-terminal tail affects the rate of O⁶-BG repair is unknown although it is possible that this tail may indirectly affect the formation
of the MGMT active site structure needed to properly orient MGMT around bulkier molecules such as O\textsuperscript{6}-BG. In this case, repair of O\textsuperscript{6}-BG by a full-length Ada fused to the carboxyl-terminal tail may be expected to occur assuming the ability of the tail alone to alter the formation of Ada's active site pocket in such a way as to allow for binding to O\textsuperscript{6}-BG. To address this possibility, active purified MGMT, Ada, and ADA-MGMTTAIL fusion proteins (1.6 pmol) were analyzed for their ability to repair O\textsuperscript{6}-BG by incubating these proteins with a 10-fold molar excess of O\textsuperscript{6}-BG for varying times. These proteins were subsequently incubated with the 18-bp DNA substrate for 2 h. O\textsuperscript{6}-BG mediated inhibition of O\textsuperscript{6}-MG repair was assessed using the restriction endonuclease inhibition assay. Ada and ADA-MGMTTAIL were unaffected by O\textsuperscript{6}-BG pre-exposure (100µM) (Figure 19, 0-10 min) whereas MGMT was completely inactivated within 2 min. The inability of ADA-MGMTTAIL to repair O\textsuperscript{6}-BG indicates that the MGMT 28 aa carboxyl-terminal tail alone is insufficient to alter Ada's active site in such a way as to facilitate the repair of O\textsuperscript{6}-BG.

C. Analysis of MGMT Amino Acids Required for the Repair of O\textsuperscript{6}-MG in DNA

To identify the minimum aa sequence required for MGMT repair activity, a series of MGMT oligopeptides and amino- or carboxyl-terminal MGMT deletion proteins were assessed for
Figure 19. Analysis of repair of O\textsuperscript{6}-BG by the ADA-MGMTTAIL fusion protein. MGMT (closed triangle), Ada (closed circle), and ADA-MGMTTAIL (open triangle) fusion proteins (1.6 pmol) were reacted in triplicate with 16 pmol of O\textsuperscript{6}-BG for 0-10 min at 37°C. These proteins were subsequently incubated with a \textsuperscript{32}P-end-labeled O\textsuperscript{6}-MG-containing DNA substrate for 2 h. O\textsuperscript{6}-BG-mediated inhibition of O\textsuperscript{6}-MG repair was monitored by the restriction endonuclease inhibition assay as described in figure 13.
Figure 20. Repair of $\text{O}^6$-MG in DNA by control and truncated MGMT fusion proteins and oligopeptides. Sonicates (50µg of total protein) of nontransformed ada$^\text{agt}^-$ cells (lane 4) or of ada$^\text{agt}^-$ cells transformed with MGMT cDNA constructs encoding either MGMT-C65, MGMT, MGMT-C35, MGMT-N18, or MGMT-N78 fusion proteins (lanes 5-9, respectively) did not contain detectable levels of alkyltransferase activity. Furthermore, none of the MGMT mutant fusion proteins or oligopeptides were able to repair alkylation damage in DNA. Additionally, sonicates of bacteria transformed with MGMT cDNA constructs encoding MGMT-C35, MGMT-N18, or MGMT-N78 (Figure 20, lanes 7, 9, 11) did not contain detectable levels of alkyltransferase activity.
their ability to repair $O^6$-MG in DNA. Figure 20 represents an autoradiograph from an alkyltransferase assay in which the $^{32}$P-end-labeled $O^6$-MG-containing 18-bp DNA substrate was cleaved with $PvuII$ following reaction with sonicates containing 50µg of total protein. Sonicates of ada$^{-}ogt^{-}$ cells transformed with MGMT cDNA constructs encoding either MGMT-C65, MGMT, MGMT-C35, MGMT-N18, or MGMT-N78 fusion proteins or 25 µg of purified MGMT Peptide 1, Peptide 2, Peptide 3, or Peptide 4 (lanes 5-13, respectively) were assayed. Alkyltransferase activity was present in sonicates containing MGMT (Figure 20, lane 6) but was absent in nontransformed ada$^{-}ogt^{-}$ cell sonicates (Figure 20, lane 4) and in sonicates containing MGMT-C65 which lacks sequences encoding the PCHRV acceptor site (Fig 16, lane 5). MGMT Peptides 1-4 (Figure 20, lanes 10-13, respectively), which do contain the PCHRV acceptor site, were also inactive. Additionally, sonicates of bacteria transformed with MGMT cDNA constructs encoding MGMT-C35, MGMT-N18, or MGMT-N78 (Figure 20, lanes 7,8,9, respectively) did not contain detectable levels of alkyltransferase activity. Furthermore, none of the MGMT mutant fusion proteins or oligopeptides were able to repair $O^6$-MG in DNA even upon prolonged exposure (18 h) of the DNA substrate in a 50-fold molar protein excess (data not shown). Truncation of MGMT did not, however, appear to affect protein expression or stability since all purified deletion mutants were expressed at levels comparable to the wildtype protein.
and were of the predicted size as demonstrated by SDS-PAGE analysis.

While studies of MGMT oligopeptides and deletion proteins suggest that nearly the entire MGMT protein is required for O\textsuperscript{6}-MG repair in DNA, the possibility exists that these mutants are capable of repairing O\textsuperscript{6}-MG in DNA, but have lost sequences which allow for the interaction of the protein with DNA prior to DNA repair. In this scenario, repair by the MGMT deletion fusion proteins and oligopeptides would be expected to be low or absent given the inability of these proteins to interact with the substrate in anything but a random fashion. To address this possibility, deletion MGMT fusion proteins and oligopeptides were analyzed for their ability to repair a damaged free base. Although O\textsuperscript{6}-MG is a logical choice, the free base chosen, O\textsuperscript{6}-BG, is a better substrate for MGMT being repaired 1000-fold more efficiently than the free base O\textsuperscript{6}-MG (Dolan et al., 1991). Since repair of O\textsuperscript{6}-BG by even the wildtype MGMT is independent of DNA interaction, truncated MGMT proteins and oligopeptides capable of repair but incapable of DNA binding, would be expected to repair this substrate. As shown in Table 1, incubation of bacterial sonicates containing an approximate 100-fold molar excess of the full-length MGMT fusion protein with 8-[\textsuperscript{3}H]-O\textsuperscript{6}-benzylguanine produced nearly complete conversion to 8-[\textsuperscript{3}H]guanine (99.5\%±1.5) as measured by reverse phase HPLC separation of the compounds. In contrast,
Table 1. Analysis of O\(^6\)-BG repair activity by control MGMT, Ada, or truncated MGMT fusion proteins and oligopeptides.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>MGMT</td>
<td>99.5 ± 1.5</td>
<td>0.5 ± 1.5</td>
</tr>
<tr>
<td>Ada</td>
<td>0.0 ± 1.0</td>
<td>100.0 ± 1.0</td>
</tr>
<tr>
<td>ada· ogt(^c)</td>
<td>0.0 ± 1.0</td>
<td>100.0 ± 1.0</td>
</tr>
<tr>
<td>MGMT-N18</td>
<td>0.0 ± 2.0</td>
<td>100.0 ± 2.0</td>
</tr>
<tr>
<td>MGMT-N78</td>
<td>0.2 ± 2.0</td>
<td>99.8 ± 2.0</td>
</tr>
<tr>
<td>MGMT-C35</td>
<td>0.0 ± 1.0</td>
<td>100.0 ± 1.0</td>
</tr>
<tr>
<td>Peptide 1</td>
<td>0.2 ± 1.5</td>
<td>99.8 ± 1.5</td>
</tr>
<tr>
<td>Peptide 2</td>
<td>0.0 ± 1.0</td>
<td>100.0 ± 1.0</td>
</tr>
<tr>
<td>Peptide 3</td>
<td>0.2 ± 1.2</td>
<td>99.8 ± 1.2</td>
</tr>
<tr>
<td>Peptide 4</td>
<td>0.2 ± 1.5</td>
<td>99.8 ± 1.5</td>
</tr>
</tbody>
</table>

11.5 pmol of 8-[^3]H]-O\(^6\)-benzylguanine were incubated in triplicate with a 100-fold molar excess of MGMT, Ada, MGMT-N18, MGMT-N78, and MGMT-C35 fusion proteins and MGMT Peptides 1-4 for 2 h at 37\(^\circ\)C. Measurements of 8-[^3]H]guanine formation from 8-[^3]H]-O\(^6\)-benzylguanine for each sample were determined by reverse phase HPLC separation of the compounds. A control incubation of non-transformed ada·ogt\(^c\) bacterial sonicates (50µg) with the O\(^6\)-BG substrate was carried out for the same time in the same buffer.
incubation of the O⁶-BG substrate with ada−ogt− bacterial sonicates produced no significant 8-[³H]guanine (0.0% ±1.0). Sonicates containing an estimated 100-fold molar excess of either ADA, MGMT-N18, MGMT-N78, or MGMT-C35 fusion proteins and MGMT Peptides 1-4 also mediated no significant repair of O⁶-BG. Combined with the results from figure 20, these studies demonstrate that the 22 aa immediately surrounding the PCHR acceptor site alone are insufficient for O⁶-MG repair in DNA and, in connection with the carboxyl- and amino-terminal deletion analyses, suggest that O⁶-MG repair in DNA requires nearly the entire protein. Furthermore, results from Table 1 suggest that the inability of the above described MGMT oligopeptides and deletion fusion proteins to repair O⁶-MG-containing DNA is not merely due to an inability to interact with DNA, but to an inability to recognize and/or repair guanine damaged at the O⁶-position as a free base or in the context of DNA. These studies, therefore, suggest that MGMT functions as a single domain protein responsible for DNA repair activity.

D. Analysis of MGMT Amino Acid Regions Involved in O⁶-Benzyguanin Substrate Specificity

1. Repair of O⁶-MG in DNA by control MGMT, Ada, or MGMT-ADA and ADA-MGMT chimeric fusion proteins

While analysis of aa involved in MGMT-mediated repair of O⁶-MG in DNA contributes to a better understanding of how alkyltransferases function, an understanding of how MGMT
differentially interacts with various lesions such as O\textsuperscript{6}-BG is lacking. The identification, therefore, of MGMT aa potentially involved in O\textsuperscript{6}-BG substrate specificity has been accomplished by assessing the ability of a series of chimeric proteins containing both MGMT and Ada sequences to repair O\textsuperscript{6}-BG. Prior to the assessment of MGMT aa regions involved in the differential repair of O\textsuperscript{6}-BG, however, MGMT-ADA and ADA-MGMT chimeras were first analyzed to verify their ability to repair O\textsuperscript{6}-MG in DNA. The ADA-PCHRV-MGMT and MGMT-PCHRV-ADA chimeric fusion proteins, consisting of MGMT and Ada sequences fused at the PCHRV acceptor site, were constructed to analyze the role of sequences carboxyl-terminal to the PCHRV acceptor site in O\textsuperscript{6}-BG repair. The ADA-LELS-MGMT and MGMT-GLHE-ADA chimeric proteins, consisting of MGMT and Ada sequences fused at the MGMT amino-terminal LELS or GLHE sequences, respectively, were constructed to analyze the role of sequences amino-terminal to the PCHRV acceptor site in O\textsuperscript{6}-BG repair. To determine whether these chimeras are active proteins, an alkyltransferase activity assay was used. Figure 21 represents an autoradiograph from an alkyltransferase assay in which the \textsuperscript{32}P-end-labeled O\textsuperscript{6}-MG-containing 18-bp DNA substrate was cleaved with PvuII following reaction with sonicates (50\mu g of total protein) of non-transformed ada\textsuperscript{ogt} cells or of ada\textsuperscript{ogt} cells transformed with MGMT, ada, or chimeric cDNA constructs encoding either MGMT, Ada, ADA-PCHRV-MGMT, or MGMT-PCHRV-ADA
fusion proteins. Alkyltransferase activity was absent in non-transformed ada·o -t· cell sonicates (Figure 21, lane 4) but was present in sonicates containing MGMT, Ada, ADA-PCHRV-MGMT, or MGMT-PCHRV-ADA fusion proteins (Figure 21, lanes 5-8, respectively). These results demonstrate that alkyltransferases from divergent evolutionary backgrounds can be fused together at the PCHRV acceptor site and still remain active, suggesting that the protein conformation which mediates O6-MG repair is comparable or at least complementary in both proteins. As described in section B, the unique MGMT 28 aa carboxyl-terminal tail is not required for MGMT activity as demonstrated by the ability of the MGMT-PCHRV-ADA protein, which lacks the tail, to repair O6-MG in DNA. The aa region between the PCHRV acceptor site and the MGMT carboxyl-terminal tail, however, is necessary for activity as demonstrated (in section C) by the inability of the MGMT-C35 deletion mutant to repair O6-MG-containing DNA. This region, however, appears to be functionally interchangeable between the bacteria and human alkyltransferases as both MGMT-PCHRV-ADA and ADA-PCHRV-MGMT fusion proteins exhibited DNA repair activity. The activity of chimeric proteins fused at amino-terminal sequences was also determined using a similar alkyltransferase activity assay in which the 18-bp DNA substrate was reacted with increasing µg amounts of total protein (0-200µg) derived from sonicated, IPTG-induced
Figure 21. Repair of O\textsuperscript{6}-MG in DNA by control MGMT, Ada, or MGMT-PCHRV-ADA, and ADA-PCHRV-MGMT fusion proteins. Sonicates (50\mu g of total protein) of nontransformed ada\textsuperscript{ogt} cells (lane 4) or of ada\textsuperscript{ogt} cells transformed with MGMT, Ada, or chimeric cDNA constructs encoding either MGMT, Ada, ADA-PCHRV-MGMT, or MGMT-PCHRV-ADA fusion proteins (lanes 5-8, respectively) were analyzed in triplicate for alkyltransferase repair activity using the restriction endonuclease inhibition assay as described in Figure 13.
bacteria containing either ADA-LELS-MGMT or MGMT-GLHE-ADA fusion proteins. Alkyltransferase activity was readily detectable using as little as 2µg total protein bacterial sonicates containing ADA-LELS-MGMT or MGMT-GLHE-ADA fusion proteins (Figures 22 and 23, respectively). The repair of O6-MG-containing DNA substrate by these proteins increased to 23±5.0 of control values when 5µg ADA-LELS-MGMT total protein (Figure 22) or 10µg MGMT-GLHE-ADA total protein (Figure 23) was used. Both chimeric proteins, however, exhibited only a 30% maximal repair capacity relative to the control MGMT, even when up to 200µg of the protein were assayed. This minimal repair was not likely due to the instability of the proteins since all fusion mutants were expressed to levels comparable to the wildtype and were of the predicted size as demonstrated by SDS-PAGE analysis. Combined with the results from figure 21 these studies demonstrate that while some aa sequences which mediate protein activity are complementary in both proteins, the amino-terminal regions of MGMT and Ada contain non-interchangeable aa that, if replaced, alter the ability of the protein to repair O6-MG in DNA.

2. Quantitation of active control and chimeric Ada and MGMT fusion proteins

To identify MGMT aa regions involved in the differential repair of O6-BG, amounts of active purified chimeric fusion
Figure 22. Repair of O\textsuperscript{6}-MG in DNA by ADA-LELS-MGMT chimeric fusion protein. Sonicates containing ADA-LELS-MGMT fusion proteins (0-200µg total protein) were assayed for alkyltransferase repair activity by reaction in triplicate with a \textsuperscript{32}P-end-labeled O\textsuperscript{6}-MG-containing DNA substrate for 2 h at 37°C.
Figure 23. Repair of $\text{O}^6\text{-MG}$ in DNA by MGMT-GLHE-ADA chimeric fusion protein. Sonicates containing MGMT-GLHE-ADA fusion proteins (0-200µg total protein) were assayed for alkyltransferase repair activity by reaction in triplicate with a $^{32}$P-end-labeled $\text{O}^6\text{-MG}$-containing DNA substrate for 2 h at $37^\circ\text{C}$.
protein were first determined using an alkyltransferase activity assay in which 1.6 pmol of MGMT, MGMT-PCHRV-ADA, Ada, or ADA-PCHRV-MGMT fusion protein (as determined by densitometric scanning of the Coomassie blue stained gel containing the control and chimeric MGMT and Ada fusion proteins) were reacted with 1.0-2.2 pmol of $^{32}$P-end-labeled O$_6$-MG-containing DNA. Lack of complete repair by ADA-LELS-MGMT and MGMT-GLHE-ADA (Figures 22 and 23) prohibited quantitation of active proteins and, therefore, all subsequent studies with these proteins were carried out using bacterial sonicates containing 50µg total protein. At a molar excess of MGMT, MGMT-PCHRV-ADA, Ada and ADA-PCHRV-MGMT, alkyltransferase activity (expressed as percent PvuII-mediated cleavage of protein-reacted DNA relative to HaeIII-mediated cleavage of the same DNA substrate) was essentially 100% for all proteins (data not shown). The highest molar concentration of DNA substrate that was 100% repaired is equivalent to the molar amount of active fusion protein used for that given reaction. The quantitation of chimeric proteins by this functional analysis (1.4-1.5 pmol for all proteins) (data not shown) was in close agreement with that derived by densitometric scanning and Bradford analysis (1.6 pmol). These results indicate that all chimeric proteins exhibited similar activity to MGMT and Ada and given a molar excess of protein, all chimeras will completely repair O$_6$-MG in DNA.
3. Repair of O⁶-BG by MGMT-ADA and ADA-MGMT chimeric fusion proteins

To identify aa involved in MGMT-selective repair of O⁶-BG, MGMT, Ada, MGMT-PCHRV-ADA, or ADA-PCHRV-MGMT chimeric fusion proteins were indirectly analyzed for the rates of repair of O⁶-BG by incubating these chimeric proteins (1.6 pmol) for varying times with a 10-fold molar excess of O⁶-BG followed by the addition of the 18-bp DNA substrate previously described. The ability of the various proteins to repair O⁶-BG was measured as inhibition of the repair of O⁶-MG in DNA by O⁶-BG pre-incubation using the restriction endonuclease inhibition assay. MGMT was partially inactivated within seconds of O⁶-BG exposure (Figure 24, 0 time point) and was completely inactivated (as measured by inhibition of PvuII-mediated cleavage of the protein-reacted DNA, relative to non-PvuII-digested DNA) within 2 min. The time course of O⁶-BG repair by the MGMT-PCHRV-ADA fusion protein was similar to the full-length MGMT with both proteins being completely inactivated by O⁶-BG within 2 min (Figure 24) although pre-incubation for various times without O⁶-BG did not affect protein activity. Ada and ADA-PCHRV-MGMT fusion proteins, however, were insensitive to O⁶-BG inhibition (Figure 24). Furthermore, ADA-PCHRV-MGMT, when incubated with a 10-fold molar excess of O⁶-BG for a prolonged time (30 min), still remained BG insensitive (data not shown). Therefore, although both MGMT-PCHRV-ADA and ADA-
Figure 24. Analysis of repair of $O^6$-BG by the MGMT-PCHRV-ADA and ADA-PCHRV-MGMT chimeric fusion proteins. MGMT (open square), Ada (closed circle), MGMT-PCHRV-ADA (closed triangle), and ADA-PCHRV-MGMT (open triangle) purified fusion proteins (1.6 pmol) were reacted in triplicate with 16 pmol of $O^6$-BG for 0-10 min at 37°C. These proteins were subsequently incubated with a $^{32}$P-end-labeled $O^6$-MG-containing DNA substrate for 2 h. $O^6$-BG repair was measured as inhibition of the repair of $O^6$-MG in DNA by $O^6$-BG pre-incubation using the restriction endonuclease inhibition assay as described in figure 13.
PCHRV-MGMT share the same PCHRV acceptor site, and both repair O\textsuperscript{6}-MG-containing DNA, only MGMT-PCHRV-ADA repaired O\textsuperscript{6}-BG, suggesting that sequences amino terminal to the PCHRV acceptor site contribute to MGMT-mediated repair of O\textsuperscript{6}-BG. This finding is further supported by analysis of O\textsuperscript{6}-BG repair by non-purified ADA-LELS-MGMT and MGMT-GLHE-ADA which are fused at amino-terminal regions. As described, both proteins repaired O\textsuperscript{6}-MG in DNA but to a lesser extent (30%) than the control Ada or MGMT fusion proteins. This activity, however, was eliminated by pre-incubation of ADA-LELS-MGMT with 100µM O\textsuperscript{6}-BG for varying times (Figure 25) although pre-incubation for various times without O\textsuperscript{6}-BG did not affect protein activity. In contrast, repair of O\textsuperscript{6}-MG-containing DNA by MGMT-GLHE-ADA was unaffected by O\textsuperscript{6}-BG pre-exposure (Figure 25, 0-10 min), suggesting that MGMT aa between the amino-terminal GLHE region and the PCHRV acceptor site (aa 27-148) are involved in the differential repair of O\textsuperscript{6}-BG.

4. Repair of O\textsuperscript{6}-BG by MGMT-ADA-MGMT chimeric fusion proteins

To further define which MGMT aa region or regions are involved in O\textsuperscript{6}-BG substrate specificity, a series of MGMT-ADA-MGMT chimeric proteins, which as a whole contain Ada aa regions in place of MGMT aa regions 1, 2, 3, and 5 (Figure 26), were analyzed for their ability to repair O\textsuperscript{6}-MG in DNA
Figure 25. Analysis of repair of \( O^6 \)-BG by the ADA-LELS-MGMT and MGMT-GLHE-ADA chimeric fusion proteins. ADA-LELS-MGMT (closed circle) and MGMT-GLHE-ADA (open triangle) non-purified fusion proteins (50µg) were reacted in triplicate with a 100-fold molar excess of \( O^6 \)-BG for 0-10 min at 37°C. These proteins were subsequently incubated with a \(^{32}\)P-end-labeled \( O^6 \)-MG-containing DNA substrate for 2 h. \( O^6 \)-BG repair was measured as inhibition of the repair of \( O^6 \)-MG in DNA by \( O^6 \)-BG pre-incubation using the restriction endonuclease inhibition assay as described in figure 13.
and O₆-BG using the restriction endonuclease inhibition assay. Figure 27 (lanes 1-10) represents an autoradiograph from an alkyltransferase activity assay in which the 18-bp DNA substrate was cleaved with PvuII following reaction with sonicates (50µg total protein) of non-transformed ada⁻⁰ᵍᵗ⁻ cells or of ada⁻⁰ᵍᵗ⁻ cells transformed with MGMT, ada, or chimeric cDNA constructs encoding MGMT, Ada, MGMT-ADA1-MGMT, MGMT-ADA2-MGMT, MGMT-ADA3-MGMT, or MGMT-ADA5-MGMT fusion proteins. Alkyltransferase activity was present in sonicates containing MGMT, Ada, MGMT-ADA1-MGMT, MGMT-ADA2-MGMT, MGMT-ADA3-MGMT, or MGMT-ADA5-MGMT fusion proteins (Figure 27, lanes 4, 5 and 7-10, respectively) but was absent in non-transformed ada⁻⁰ᵍᵗ⁻ cell sonicates (Figure 27, lane 6). All repair proficient MGMT-ADA-MGMT proteins were subsequently analyzed for the repair of O₆-BG by pre-incubating these chimeric proteins with an estimated 10-fold molar excess of O₆-BG for 30 min followed by the addition of the O₆-MG-containing 18-bp DNA substrate for an additional 2 h. O₆-BG mediated inhibition of O₆-MG repair was assessed using the restriction endonuclease inhibition assay. MGMT, MGMT-ADA1-MGMT, MGMT-ADA2-MGMT, MGMT-ADA3-MGMT, and MGMT-ADA5-MGMT were all inactivated by O₆-BG pre-exposure as shown by the lack of alkyltransferase activity in sonicates containing these chimeras (Figure 27, lanes 11 and 13-16, respectively) although pre-incubation for 30 min without O₆-BG did not affect chimeric protein activity (data not shown).
Figure 26. Summary diagram of the construction of MGMT-ADA-MGMT chimeric proteins. MGMT-ADA-MGMT chimeric fusion proteins contain Ada aa in place of either MGMT aa region 1 (MGMT-ADA1-MGMT), MGMT aa region 2 (MGMT-ADA2-MGMT), MGMT aa region 3 (MGMT-ADA3-MGMT), or MGMT aa region 5 (MGMT-ADA5-MGMT). MGMT aa regions 1-5 (bold lines) span from the MGMT amino-terminal GLHE region to the PCHRV acceptor site (bordered by arrows). Alignment of the numbered MGMT aa is according to Santibanez-Koref et al., 1992.
Figure 27. Repair of O⁶-MG in DNA and O⁶-BG by control MGMT, Ada, and MGMT-ADA-MGMT chimeric fusion proteins. Sonicates (50µg total protein) of non-transformed ada-ogt⁻ cells (lane 6) or of ada⁻ogt⁻ cells transformed with MGMT, ada, or chimeric cDNA constructs encoding either MGMT, Ada, MGMT-ADA1-MGMT, MGMT-ADA2-MGMT, MGMT-ADA3-MGMT, or MGMT-ADA5-MGMT fusion proteins (lanes 4, 5, and 7-10, respectively) were analyzed in triplicate for alkyltransferase repair activity using the restriction endonuclease inhibition assay as described in figure 13. Sonicates (50µg total protein) of ada⁻ogt⁻ cells transformed with MGMT, ada, or chimeric cDNA constructs encoding either MGMT, Ada, MGMT-ADA1-MGMT, MGMT-ADA2-MGMT, MGMT-ADA3-MGMT, or MGMT-ADA5-MGMT fusion proteins (lanes 11-16, respectively) were reacted in triplicate with a 10-fold molar excess of O⁶-BG for 30 min followed by the addition of the ³²P-end-labeled O⁶-MG-containing DNA substrate for an additional 2 h. O⁶-BG repair was measured as inhibition of the repair of O⁶-MG in DNA by O⁶-BG pre-incubation using the restriction endonuclease inhibition assay.
In contrast, Ada was proficient at the repair of O⁶-MG in DNA even after O⁶-BG exposure (Figure 27, lane 12). The ability of all MGMT-ADA-MGMT chimeras to repair O⁶-BG suggests that MGMT aa regions 1, 2, 3, and 5 alone (Figure 26) are not involved in MGMT-mediated repair of O⁶-BG. By inference, therefore, the unique ability of MGMT to interact with O⁶-BG appears to reside in any of 49 aa spanning three MGMT aa regions amino-terminal to the PCHRV acceptor site (Figure 28).

E. Comparison of Ada Amino Acid Regions Involved in O⁴-Methylthymine Substrate Specificity to that of MGMT Amino Acid Regions Involved in O⁶-Benzylguanine Substrate Specificity

Differences in MGMT and Ada substrate specificity can be further defined by comparing Ada aa regions involved in the repair of O⁴-MT in DNA to MGMT aa regions involved in the repair of O⁶-BG. The differences between MGMT and Ada in the repair of O⁶-BG and O⁴-MT may be accounted for by a few common aa changes within MGMT and Ada or, alternatively, MGMT and Ada may differ significantly in the conformation of their active sites such that different aa regions account for MGMT-mediated repair of O⁶-BG and Ada-mediated repair of O⁴-MT. To compare aa regions involved in O⁶-BG substrate specificity to those involved in O⁴-MT substrate specificity, MGMT, Ada, MGMT-PCHRV-ADA, or ADA-PCHRV-MGMT fusion proteins (100-fold molar excess) were analyzed for their ability to repair O⁴-
Figure 28. Summary diagram of MGMT aa regions potentially involved in MGMT-mediated repair of $O^6$-BG. Solid black lines indicate $O^6$-BG specificity sites. The PCHRV acceptor site is marked by the boxed area. Alignment of the numbered MGMT aa is according to Santibanez-Koref et al., 1992.
methyl[^3]H]thymine (approximately 0.5 pmol) in poly(dA)-poly(dT) substrate. O^4-MT repair was assessed by comparing O^4-methyl[^3]H]thymine content in poly(dA)-poly(dT) exposed to full-length or chimeric MGMT and Ada proteins to that in control poly(dA)-poly(dT) substrate. As described previously, (Dolan et al., 1984) Ada significantly reduced O^4-MT content relative to incubations containing polymer alone as measured by reverse phase HPLC (Table 2). Furthermore, the repair was specific for O^4-MT as no change was observed in 3-methylthymine content following protein incubation. There was no significant repair of O^4-MT, however, when MGMT, MGMT-PCHRV-ADA, and ADA-PCHRV-MGMT were incubated with the O^4-methyl[^3]H]thymine-containing poly(dA)-poly(dT) substrate, suggesting that sequences surrounding PCHR V are relevant to Ada-mediated O^4-MT repair. In a parallel experiment in which the same protein preparations and buffer conditions were used, all proteins were analyzed for their ability to repair O^6-MG in DNA using the alkyltransferase assay. Figure 29 represents an autoradiograph from an alkyltransferase activity assay in which the 18-bp DNA substrate was cleaved with PvuII following reaction with sonicates of non-transformed ada^-ogt^- cells or of ada^-ogt^- cells transformed with MGMT, Ada, or chimeric cDNA constructs encoding Ada, MGMT, ADA-PCHRV-MGMT, or MGMT-PCHRV-ADA fusion proteins. Alkyltransferase activity was present only in sonicates containing Ada, MGMT, ADA-
PCHRV-MGMT, or MGMT-PCHRV-ADA fusion proteins (Figure 29, lanes 5-8, respectively). These results clearly indicate that MGMT, MGMT-PCHRV-ADA, and ADA-PCHRV-MGMT do not repair O⁴-MT under conditions in which O⁶-MG is repaired rapidly. While MGMT sequences potentially involved in O⁶-BG repair include aa amino-terminal to the PCHRV acceptor site, aa involved in Ada-selective repair of O⁴-MT are both amino- and carboxyl-terminal to the PCHRV acceptor site. These studies exclude the possibility that a few common aa changes within MGMT and Ada can account for both O⁴-MT and O⁶-BG repair capacities. Instead, MGMT and Ada may differ significantly in the conformation of their active sites such that different aa regions within MGMT and Ada define the overall substrate specificity of the proteins.
Table 2. Repair of O\textsuperscript{4}-MT in DNA by MGMT-PCHRV-ADA and ADA-PCHRV-MGMT chimeric fusion proteins.

<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>METHYLATED BASES REMAINING IN SUBSTRATE (PMOL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3-METHYLTHYMINE</td>
</tr>
<tr>
<td>NONE</td>
<td>0.60 ±0.03</td>
</tr>
<tr>
<td>ADA</td>
<td>0.60 ±0.02</td>
</tr>
<tr>
<td>MGMT</td>
<td>0.63 ±0.01</td>
</tr>
<tr>
<td>ADA-PCHRV-MGMT</td>
<td>0.55 ±0.02</td>
</tr>
<tr>
<td>MGMT-PCHRV-ADA</td>
<td>0.58 ±0.01</td>
</tr>
</tbody>
</table>

N-[\textsuperscript{3}H]methyl-N-nitrosourea-exposed poly(dA)-poly(dT) substrate containing 0.5 pmol O\textsuperscript{4}-methyl[\textsuperscript{3}H]thymine and 0.6 pmol 3-methyl[\textsuperscript{3}]thymine was incubated in triplicate with a 100-fold molar excess of MGMT, Ada, MGMT-PCHRV-ADA, or ADA-PCHRV-MGMT fusion proteins for 2 h at 37°C. A control incubation without added protein was carried out for the same time in the same buffer. O\textsuperscript{4}-MT repair was determined by comparing O\textsuperscript{4}-methyl[\textsuperscript{3}H]thymine content in poly(dA)-poly(dT) exposed to full-length or chimeric MGMT and Ada proteins to that in control poly(dA)-poly(dT) substrate as measured by reverse phase HPLC.
Figure 29. Repair of O\textsuperscript{6}-MG in DNA by control MGMT, Ada, ADA-PCHRV-MGMT, or MGMT-PCHRV-ADA fusion proteins. Sonicates (50µg of total protein) of nontransformed ada\textsuperscript{-}ogt\textsuperscript{-} cells (lane 4) or of ada\textsuperscript{-}ogt\textsuperscript{-} cells transformed with Ada, MGMT, or chimeric cDNA constructs encoding Ada, MGMT, ADA-PCHRV-MGMT, or MGMT-PCHRV-ADA fusion proteins (lanes 5-8, respectively) were analyzed in triplicate for alkyltransferase repair activity using the restriction endonuclease inhibition repair assay as described in Figure 13.
While DNA repair by all alkyltransferases depends upon an essential acceptor cysteine located within the highly conserved PCHRV acceptor site, very little else is known concerning the contribution of other aa, both conserved and divergent, in alkyltransferase folding, stability, repair activity, and substrate specificity. The importance of the PCHRV acceptor site and other surrounding aa in alkyltransferase function, however, has been recently demonstrated by site-directed mutagenesis studies (Ling-Ling et al., 1992; Pieper et al., 1994; Rafferty et al., 1994). These studies, in addition to the presence of highly conserved or invariant aa surrounding the cysteine acceptor site, suggest that an understanding of MGMT aa required for activity and substrate specificity requires an analysis of aa beyond those directly involved in alkyl group transfer. The studies in this dissertation were therefore designed to systematically analyze the role of aa regions in protein function by generating a series of MGMT and Ada chimeric proteins and MGMT deletion mutants and oligopeptides, and examining the biochemical properties of these mutant proteins.
including repair of O⁶-MG and O⁴-MT in DNA and O⁶-BG. These studies have successfully identified aa regions which play a role in MGMT activity and substrate specificity and thus these studies, as a whole, have contributed to the overall understanding of how MGMT functions. The interpretation and significance of these findings are discussed below.

Several experiments described in this dissertation indicate that aa located throughout nearly the entire protein are involved in MGMT-mediated DNA repair activity. With regard to the MGMT oligopeptide study, the inability of the MGMT PCHRV-containing oligopeptides to repair O⁶-MG in DNA suggests that the highly conserved 22 aa immediately surrounding the PCHRV acceptor site alone are insufficient for O⁶-MG repair. This finding is in agreement with recent studies on the Ada crystal structure which suggest that a larger, highly conserved 36 aa region spanning from asparagine 138 to glutamic acid 173 surrounding the PCHRV acceptor site plays a direct role in the formation of the protein’s active site (Moore et al., 1994). In this region, there exist four absolutely conserved aa that are involved in direct or indirect hydrogen bonding to the acceptor cysteine. This region of the protein is thus believed to properly form, stabilize, and position the acceptor cysteine for nucleophilic attack and removal of alkyl lesions from the O⁶-position of guanine in DNA. This 36 aa region, however, still constitutes only a portion of the core sequences
required for alkyltransferase activity as the MGMT-N18, MGMT-N78, and MGMT-C35 deletion mutants were also unable to repair O⁶-MG in DNA. Although these truncated GST-MGMT fusion proteins were non-functional, truncation of MGMT did not appear to affect protein expression or stability since all purified deletion mutants were expressed to levels comparable to the wild type protein and were of the predicted size as demonstrated by SDS-PAGE analysis. Combined data based on the inactive MGMT oligopeptides and MGMT deletion proteins therefore suggest that O⁶-MG repair in DNA requires nearly the entire protein. Interestingly, based on the Ada crystal structure, there exists a highly conserved 85 aa carboxy-terminal structural domain while the remaining amino-terminal 88 aa of Ada comprise a second structural domain which is relatively non-conserved between alkyltransferases (Moore et al., 1994). One may thus propose initially that these two structurally independent regions may also be functionally independent domains. MGMT-N78, which lacks nearly the entire amino-terminal structural domain, however, does not repair O⁶-MG in DNA, suggesting that the proposed structural regions are not functionally independent. Additional aa regions needed for protein activity, therefore, include sequences from both the amino- and carboxyl-terminal regions.

While studies of MGMT oligopeptides and deletion proteins suggest that nearly the entire MGMT protein may be required for the repair of O⁶-MG containing DNA, the
possibility exists that these same proteins retain the capacity to repair O\textsuperscript{6}-MG in DNA, but do so less efficiently as a consequence of loss of sequences required for MGMT-DNA interaction prior to DNA repair. The mutant proteins were, however, also incapable of repairing the free base O\textsuperscript{6}-BG. The inability of the MGMT oligopeptides and deletion proteins to repair O\textsuperscript{6}-MG in DNA and O\textsuperscript{6}-BG suggests that, as discussed above, nearly the entire protein is required for MGMT-mediated DNA repair activity. Alternatively, pertaining to MGMT-DNA interaction, these data may also indirectly suggest that MGMT does not contain a separate DNA binding domain independent of a domain required for alkyltransferase activity since the MGMT truncated fusion proteins and oligopeptides were shown to not only lose the ability to repair damaged guanine in the context of DNA but also the ability to repair damaged guanine as a free base. Further understanding of how MGMT and its active site may interact with DNA can be derived from studies on the Ada crystal structure which suggest that the proposed 85 aa carboxyl-terminal structural domain, containing the cysteine acceptor site, constitutes in part a helix-turn-helix (HTH) DNA-binding motif. This putative HTH motif is proposed, but not proven, to be essential for the interaction of the protein with duplex DNA (Moore et al., 1994). DNA interaction may be important for MGMT activity since this proposed binding is believed to induce a conformational change in the protein, as
monitored by circular dichroism and fluorescence analyses (Chan et al., 1993). This conformational change may be needed to properly orient the cysteine for nucleophilic attack on target alkyl groups. Indeed, the rate determining step in the transfer of alkyl groups to MGMT molecules has been suggested to be DNA binding (Chan et al., 1994; Takahashi et al., 1990). The interaction of MGMT with duplex DNA may also be required to induce a conformational change in the O⁶-MG-containing DNA substrate. Evidence of protein-induced structural distortion of DNA substrates comes from studies on the recently cocrystallized structure of a DNA cytosine-5-methyltransferase bound covalently to DNA (Klimasauskas et al., 1994). In this structure, the cytosine residue, acting as substrate, has swung completely out of the DNA helix and is positioned in the active site of the protein which itself has undergone a conformational change. This co-crystal structure illustrates a novel mechanism as to how the active site of the protein can reach its target DNA substrate. Given the fact that the Ada crystal structure reveals a concave active site containing a deeply buried acceptor cysteine, such structural distortion of O⁶-MG-containing DNA by alkyltransferases may also apply. To further understand MGMT-DNA binding interactions, however, additional structural studies on stable protein-bound complexes with O⁶-MG-containing DNA are needed. Because the rate of repair of O⁶-MG in DNA by the wildtype MGMT is
extremely fast, the design of mutant MGMT proteins that bind DNA but do not carry out the transfer reaction would greatly facilitate these studies.

Although the studies described above indicate that MGMT is comprised of a single protein domain responsible for repair of O\textsuperscript{6}-MG-containing DNA, the possibility still remains that MGMT may be a multi-functional protein mediating non-repair related activities. Such activities may be mediated by the MGMT 28 aa carboxyl-terminal tail given the tail's evolutionary sequence conservation among human, rat, mouse, and hamster alkyltransferases and the tail's dispensability in O\textsuperscript{6}-MG repair. Specifically, studies in this dissertation and elsewhere (Elder et al., 1992) have demonstrated that up to 28 aa at the carboxyl-terminus can be deleted without loss of alkyltransferase activity. Furthermore, the rates of repair of O\textsuperscript{6}-MG in DNA mediated by the full-length MGMT and mutant MGMT-C28 were equivalent. While analysis of several unrelated proteins has also suggested that not all aa are required for intrinsic protein activity (Alber et al., 1988; Lim and Sauer, 1991; Morris and Wool, 1992), it remains unclear why a nonessential sequence such as the carboxyl-terminal MGMT tail should evolve and be conserved among higher organisms. One possibility, as mentioned above, is that this tail is a separate functional domain that mediates an activity completely unrelated to DNA repair. A similar 61 aa amino-terminal region in the human AP endonuclease 1
(HAP1), was recently reported to be dispensable for DNA repair function, but was essential for regulation of the DNA binding activity of the c-jun proto-oncogene product (Xanthoudakis et al., 1992; Walker et al., 1993; Xanthoudakis et al., 1994). The possibility exists that MGMT may also contain two nonoverlapping domains encoding distinct functions. Alternatively, the 28 aa carboxyl-terminal tail may have no significant role in protein function primarily since not all mammalian proteins, such as the recently isolated rabbit alkyltransferase (Iyama et al., 1994), contain this tail. The carboxyl-terminal tail does, however, appear to affect MGMT activity at lower temperatures since deletion of the tail reversibly inhibits the ability of MGMT to repair O6-MG in DNA at 4°C. The relevance of this observation to MGMT-mediated repair at physiological conditions remains uncertain, although the data support the idea that the tail sequence may be involved in maintenance of protein folding or stability. Further biochemical analysis of the MGMT 28 aa carboxyl-terminal tail and MGMT deletion mutants may be of use in assessing alternate MGMT functions.

While analysis of aa involved in MGMT-mediated repair of O6-MG in DNA contributes to a better understanding of how all alkyltransferases function, an understanding of how MGMT and Ada differentially interact with various lesions such as O6-BG and O4-MT in DNA is lacking. Differences in MGMT and Ada substrate specificity may be accounted for by a common
limited number of aa changes which allow MGMT to repair $\text{O}^6$-BG but not $\text{O}^4$-MT. In the simplest sense, a single aa change in MGMT might allow $\text{O}^6$-BG repair yet preclude $\text{O}^4$-MT repair. Alternatively, MGMT and Ada may differ significantly in the overall conformation of their active sites such that different aa regions account for MGMT-mediated repair of $\text{O}^6$-BG and Ada-mediated repair of $\text{O}^4$-MT. This dissertation addressed both possibilities by assessing the ability of MGMT and Ada chimeric proteins to repair $\text{O}^4$-MT in DNA and $\text{O}^6$-BG.

The assessment of $\text{O}^6$-MG, $\text{O}^6$-BG, and $\text{O}^4$-MT repair capacities by MGMT and Ada chimeric fusion proteins was instructional in identifying critical MGMT and Ada aa regions involved not only in substrate specificity but also in protein activity. With regard to protein activity, the chimeric proteins MGMT-PCHRV-ADA and ADA-PCHRV-MGMT repaired $\text{O}^6$-MG in DNA, suggesting that while MGMT and Ada differ in their evolutionary backgrounds, the protein conformation which mediates $\text{O}^6$-MG repair is comparable or at least complementary in both proteins. Although these studies were carried out using GST-chimeric fusion proteins, the full-length GST-MGMT protein exhibited DNA repair properties comparable to that of endogenous MGMT in HT-29 colon carcinoma cell extracts, suggesting that fusion of the GST peptide to MGMT does not appear to affect alkyltransferase activity. Additionally, based on the Ada crystal structure, the GST peptide appears to be fused to a proposed amino-
terminal structural domain which is independent from the protein's active site. This observation suggests that the GST peptide would not directly alter protein activity. As described earlier, the unique MGMT 28 aa carboxyl-terminal tail is not required for MGMT activity as demonstrated by the ability of the MGMT-PCHRV-ADA protein, which lacks the tail, to repair O^6-MG in DNA. A 30 aa region between the shared PCHRV acceptor site and the MGMT 28 aa carboxyl-terminal tail appears to be functionally interchangeable between Ada and MGMT as demonstrated by the activity of MGMT-PCHRV-ADA and ADA-PCHRV-MGMT fusion proteins. Combined data based on the X-ray crystal structure of Ada (Moore et al., 1994) and the active chimeric proteins fused at the acceptor site have highlighted the potential importance of this 30 aa region carboxyl-terminal to the PCHRV acceptor site in the repair of O^6-MG in DNA. As this 30 aa region has been proposed to constitute in part a random coil that serves to swivel the carboxyl-terminal alpha-helix and expose both a potential DNA binding surface and the buried active site thiol of cysteine, necessity and functional interchangeability of this region between all alkyltransferases might be expected. In addition to the active chimeric proteins fused at the highly conserved acceptor site, the MGMT-ADA-MGMT proteins, each containing approximately 10-20 Ada aa in place of MGMT aa, were also repair proficient, suggesting that, in regions examined, substitution of very small MGMT aa regions with the analogous
Ada aa does not appear to alter the protein's tertiary structure in such a way that affects alkyltransferase activity. In contrast, the fusion of MGMT and Ada sequences at the amino-terminus resulted in proteins with a limited alkyltransferase repair capacity. This minimal repair was not likely due to the instability of the proteins since all fusion mutants were expressed to levels comparable to the wild type and were of the predicted size as demonstrated by SDS-PAGE analysis. No explanation exists as to why these proteins exhibited only 30% maximal repair, however, the highly divergent amino terminal regions of MGMT and Ada clearly contain non-interchangeable aa which may play a key role in protein activity. Furthermore, additional MGMT and Ada chimeric proteins, each fused at one of several regions throughout the entire alkyltransferase, were unable to repair O\(^6\)-MG in DNA (data not shown) suggesting that, in regions examined, such fusions may produce perturbations in the chimeric protein's local secondary or tertiary structures.

With regard to substrate specificity, the unique ability of MGMT to interact with O\(^6\)-BG appears to reside, by indirect analysis, in any of 49 aa spanning three MGMT aa regions amino-terminal to the PCHRV acceptor site (aa 42-78, 93-98, and 138-143) (Figure 28). The 6 aa region 138-143, is of particular interest primarily because other studies have demonstrated that a single change in a proline residue (P140) located within this region reduced the ability of MGMT to
repair O⁶-BG (Crone and Pegg, 1993). This single aa change did not, however, render MGMT completely insensitive to O⁶-BG whereas the ADA-PCHRV-MGMT protein described in this dissertation was completely O⁶-BG insensitive even after prolonged incubation time of 30 minutes. These studies suggest therefore that other aa besides P140 may play a role in the formation of the MGMT active site needed for O⁶-BG repair. It is interesting to note that two of the three MGMT aa regions involved in O⁶-BG specificity appear to be proline-rich areas. Proline residues are known to constrain the conformation of adjacent residues which can lead to bends in the protein’s secondary structural elements (Crone and Pegg, 1993). Such bends may serve as extremely flexible areas of the protein which in turn may alter the overall protein conformation in such a way that creates an appropriate sized active site needed for MGMT-mediated repair of O⁶-BG.

While MGMT sequences potentially involved in O⁶-BG repair include three defined aa regions amino-terminal to the PCHRV acceptor site, aa involved in Ada-selective repair of O⁴-MT are located both amino- and carboxyl-terminal to the PCHRV acceptor site. These studies exclude the possibility that a few common aa changes within MGMT and Ada can account for both O⁴-MT and O⁶-BG repair capacities. Instead, MGMT and Ada may differ significantly in the conformation of their active sites such that different aa regions within MGMT and
Ada contribute to the protein's overall substrate specificity.

The exact mechanism by which MGMT and Ada sequences aid in MGMT-mediated repair of $O^6$-BG or Ada-mediated repair of $O^4$-MT is unclear. It is possible that divergent aa may constitute either a well-defined aa segment that borders the protein's active site or, alternatively, these aa may be scattered throughout the protein. These scattered aa, as a whole, may comprise the appropriate size pocket for differential lesion repair. With regard to scattered aa involved in substrate specificity, X-ray crystallographic studies of dihydrofolate reductase and the human butyrylcholinesterase have demonstrated that a few highly conserved aa, spread throughout the primary protein sequence, can come together in a three-dimensional structure to form an active site (Harel et al., 1992; Dicker et al., 1993). Although these essential aa were shown to be considerably distant from the enzyme's catalytic center, they appeared to affect substrate specificity by indirectly affecting the protein's tertiary structure and by facilitating in the closure of the protein's active site around the substrate. Such conformational changes induced by aa or aa regions, subsequent to protein-substrate binding, have been well studied (Dicker et al., 1993). Specific MGMT aa regions 42-78, 93-98, and 138-143, as discussed above, may contain critical aa, not present in Ada, which play a role in
increasing the size of the space surrounding the acceptor cysteine, thereby accommodating MGMT interaction with larger molecules such as O\textsuperscript{6}-BG. The MGMT 28 aa carboxyl-terminal tail region may also play a role in the formation of the MGMT acceptor site. Evidence for this notion comes from studies, described in this dissertation, which demonstrated that removal of the tail from MGMT decreased the rate of MGMT-mediated O\textsuperscript{6}-BG repair at least 5-fold, suggesting that the carboxyl-terminal tail indirectly affects O\textsuperscript{6}-BG repair activity. The presence of a tail may induce changes in the tertiary structure of the protein needed to properly form and orient the MGMT active site around bulkier molecules such as O\textsuperscript{6}-BG. Alternatively, the tail may simply be involved in positioning the PCHRV acceptor site so as to allow optimal DNA repair. The inability of ADA-MGMTTAIL to repair O\textsuperscript{6}-BG, however, indicates that the tail alone is insufficient to directly alter the protein's active site in such a way as to facilitate O\textsuperscript{6}-BG repair.

In contrast to scattered aa or aa regions playing a role in the formation of a unique acceptor site necessary for selective repair of O\textsuperscript{6}-BG or O\textsuperscript{4}-MT, the possibility also exists that MGMT and Ada, while both containing a similar sized active site pocket, contain a well-defined aa region within the active site which may confer selectivity for O\textsuperscript{6}-BG or O\textsuperscript{4}-MT substrates. Recent crystallographic studies of the Ada protein have at least indirectly suggested such an aa
region which may define the ability of the protein to recognize distinct substrates. In these studies, four aa between Asn 138 and Glu 173 directly or indirectly hydrogen bond to the acceptor cysteine suggesting that this region of the protein may form the active site of the molecule (Moore et al., 1994). It is therefore possible that this core region alone may dictate MGMT’s ability to repair $O^6$-BG (and exclude $O^4$-MT) whereas the analogous aa region in Ada may be enough to confer $O^4$-MT selectivity. Discrimination between substrates by exclusion from the protein’s acceptor site of the non-preferred substrate has been studied in other proteins (Liu and Santi, 1993). Such exclusion is a consequence of disruptive hydrogen bonding between specific aa side chain groups and the unsuitable DNA substrate. The concept of substrate acceptance versus exclusion may also be useful in understanding the action of MGMT as substrate specificity may reside in only a few aa selective for recognition and repair of $O^6$-BG. Site-directed mutagenesis studies of divergent MGMT and Ada aa, however, may lead to a better understanding of the exact mechanism by which MGMT and Ada sequences play a direct and/or indirect role in substrate specificity.

Although the general mechanism involving the transfer of alkyl groups from the $O^6$-position of guanine to a cysteine contained within the alkyltransferase’s PCHRV acceptor site is known, very little else is understood about how regions of
the MGMT primary aa sequence contribute to alkyltransferase stability, activity, and substrate specificity. The studies described in this dissertation suggest that nearly all aa contribute to MGMT-mediated repair of O^6-MG in DNA. The unique MGMT 28 aa carboxyl-terminal tail, however, was not required for the repair of O^6-MG in DNA or O^6-BG although deletion of the tail reversibly inhibited the ability of MGMT to repair O^6-MG-containing DNA at lower temperatures. With regard to substrate specificity, the unique ability of MGMT to interact with O^6-BG appears to reside in any of 49 aa spanning three MGMT aa regions amino-terminal to the PCHRV acceptor site. From these conclusions, one can suggest that MGMT is most likely a single domain protein, perhaps requiring most of its aa sequences for proper protein folding, DNA interaction, and activity, while select MGMT aa, that are either necessary or dispensable for protein activity, may play a vital role in conferring DNA substrate specificity.


Teo, I., B. Sedgwick, B. Demple, B. Li, and T. Lindahl. 1984. Induction of resistance to alkylating agents in *E. coli*: the Ada gene product serves both as regulatory protein and as an enzyme for repair of mutagenic damage. EMBO J. 3: 2151-2157.


VITA

The author, Susan E. Morgan, was born in Waterbury, Connecticut on May 22, 1968 to John and Carole Morgan.

In August, 1986, Ms. Morgan entered Ripon College in Ripon, Wisconsin, and received a Bachelor of Arts in Biology with a minor in Chemistry in May 1990. That year, she enrolled in the Program in Molecular Biology at Loyola University Chicago, Maywood, Illinois. She joined the laboratory of Russell O. Pieper, Ph.D., in April 1991, where she studied the structural and functional properties of a human DNA repair protein, O-6-methylguanine-DNA methyltransferase. In 1993, Ms. Morgan was awarded a fellowship by the Arthur J. Schmitt Foundation.

Ms. Morgan has accepted a position as a post-doctoral fellow in the laboratory of Michael B. Kastan, Ph.D., M.D., at Johns Hopkins University, Baltimore, Maryland.
ABSTRACTS


This dissertation submitted by Susan E. Morgan has been read and approved by the following committee:

Russell O. Pieper, Ph.D., Director
Assistant Professor
Department of Medicine and Pharmacology
Loyola University Chicago

M. Eileen Dolan, Ph.D.
Assistant Professor
Division of Hematology/Oncology
University of Chicago Medical Center

Leonard C. Erickson, Ph.D.
Professor
Department of Medicine and Pharmacology
Loyola University Chicago

Mark R. Kelley, Ph.D.
Associate Professor
Department of Pediatrics
Indiana University School of Medicine

John M. Lopes, Ph.D.
Assistant Professor
Department of Molecular and Cellular Biochemistry
Loyola University Chicago

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is, therefore, accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

9.6.94
Date

Russell O. Pieper
Director’s Signature