INFORMATION TO USERS

This reproduction was made from a copy of a document sent to us for microfilming. While the most advanced technology has been used to photograph and reproduce this document, the quality of the reproduction is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help clarify markings or notations which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure complete continuity.

2. When an image on the film is obliterated with a round black mark, it is an indication of either blurred copy because of movement during exposure, duplicate copy, or copyrighted materials that should not have been filmed. For blurred pages, a good image of the page can be found in the adjacent frame. If copyrighted materials were deleted, a target note will appear listing the pages in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed, a definite method of "sectioning" the material has been followed. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.

4. For illustrations that cannot be satisfactorily reproduced by xerographic means, photographic prints can be purchased at additional cost and inserted into your xerographic copy. These prints are available upon request from the Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases the best available copy has been filmed.

University Microfilms International
300 N. Zeeb Road
Ann Arbor, MI 48106
Duran, Harry Leo

THE SITE SPECIFIC MUTAGENIC EFFICIENCY OF THE ALKYLATED DNA BASE, OXYGEN-4-ETHYLTHYMINE: INTERACTIONS OF DEOXYNUCLEOTIDE TRIPHOSPHATES, POLYMERASES AND REPAIR ENZYMES IN GAP MISREPAIR MUTAGENESIS

The Ohio State University

Ph.D. 1985

University Microfilms International 300 N. Zeeb Road, Ann Arbor, MI 48106

Copyright 1985 by Duran, Harry Leo

All Rights Reserved
PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark √.

1. Glossy photographs or pages ______
2. Colored illustrations, paper or print ______
3. Photographs with dark background ______
4. Illustrations are poor copy ______
5. Pages with black marks, not original copy ______
6. Print shows through as there is text on both sides of page ______
7. Indistinct, broken or small print on several pages ______
8. Print exceeds margin requirements ______
9. Tightly bound copy with print lost in spine ______
10. Computer printout pages with indistinct print ______
11. Page(s) ________ lacking when material received, and not available from school or author.
12. Page(s) ________ seem to be missing in numbering only as text follows.
13. Two pages numbered 141_. Text follows.
14. Curling and wrinkled pages ______
15. Dissertation contains pages with print at a slant, filmed as received ______
16. Other ____________________________________________
    ____________________________________________
    ____________________________________________

University
Microfilms
International
THE SITE SPECIFIC MUTAGENIC EFFICIENCY OF THE ALKYLATED DNA BASE, O\(^{4}\)-ETHYLTHYMINE: INTERACTIONS OF DEOXYNUCLEOTIDE TRIPHOSPHATES, POLYMERASES AND REPAIR ENZYMES IN GAP MISREPAIR MUTAGENESIS

DISSERTATION

Presented In Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy In the Graduate School of The Ohio State University

by

Harry Leo Duran, B.S., M.S.

The Ohio State University

1985

Dissertation Committee:

S.M. D'Ambrosio, Ph.D.
A.A. Wani, Ph.D.
R.W. Trewyn, Ph.D.
R.M. Snapka, Ph.D.

Approved by

[Signature]

Adviser
Department of Pharmacology
Copyright by
Harry Leo Duran
1985
To Mother and Father
I wish to express my gratitude to Professor Steven M. D'Ambrosio for his support, guidance, and insight throughout this research. Thanks also to Dr. Altaf A. Wani for his input into the development and direction of this research, and for sharing his knowledge of molecular biology and site specific mutagenesis with me. I am grateful to Dr. Robert M. Snapka for his insight and input into the development of this project. Dr. Ron Trewyn was a valuable member of my dissertation committee and an engaging instructor. I am especially thankful for his wisdom in clarifying some important aspects of nucleic acid biochemistry for me. I am also appreciative of the efforts Dr. James Blakeslee and Dr. Linda Carter for teaching me valuable skills in viral carcinogenesis. Thanks to Ruth E. Gibson-D'Ambrosio for instructing me in tissue culturing techniques. The members of the Pharmacology graduate faculty; Dr. Philip Hollander, Dr. Richard Fertel, Dr. Sarah Tjloe, Dr. Gopi Tejwani, Dr. Nicholas Gerber, Dr. Daniel Courl, and Dr. Norton Neff were all valued instructors. I wish to acknowledge my classmates, George Cox, Tapu Dutta-Choudhury, Jason Chang, Jenni Climie, and Cathy Mahle and thank them for their friendship during my studies. The many hours of study which we all shared was important to me as well as the good times. My studies at this university would not have been possible without the
financial support which I received from the Ohio State University Graduate School which provided me with a Graduate Fellowship; the National Institute of General Medical Sciences, which provided me with a MARC Predoctoral Fellowship; the Bennett Fund, which was a source of stipend, and the research resources which were provided by Dr. D'Ambrosio and Dr. Wanl. Special thanks to my friend, Roger Hissong whose invaluable assistance in helping to prepare this manuscript was essential in its successful completion.
VITA

August 24, 1956 .......................... Date of Birth.

Farmington, New Mexico ........................ Place of Birth.

May, 1974 ........................................... Honor Graduate, Aztec High School, Aztec, New Mexico.

May, 1980 ........................................... Bachelor of Science degree awarded, Biology, New Mexico State University, Las Cruces, New Mexico.

December, 1982 ................................. Master of Science degree awarded, Biology (Physiology), New Mexico State University, Las Cruces, New Mexico.

September, 1982– August, 1985 ............ Doctoral student, Department of Pharmacology, The Ohio State University, Columbus, Ohio.

PUBLICATIONS


FIELDS OF STUDY

Major Field: Pharmacology

1982  Drug metabolism studies of indoprofen, and clinical pharmacologic methodology. Dr. Nicolas Gerber, Department of Pharmacology, OSU.

1983  Mass spectroscopic analysis and gas chromatography of human fluids as a method of detecting drugs and drug metabolites; clinical toxicology. Dr. Brian Andresen, Department of Pharmacology, OSU.

June, 1983 - August, 1985  Studies in site specific mutagenesis, and gene expression using molecular biologic techniques. Dr. Steven D'Ambrosio, Department of Pharmacology and Department of Radiology, OSU and Dr. Altaf A. Wani, Department of Radiology, OSU. Viral carcinogenesis studies of feline sarcoma virus, and HTLV-I in human and primate tissues, Dr. James Blakeslee, Veterinary Pathobiology, OSU.

HONORS

1974  New Mexico Institute of Mining and Technology Regents Scholarship.

1975  American Legion Scholarship.

1979-1980  NIGMS-MARC Undergraduate Honors Research Trainee, New Mexico State University.

1980  Minority Graduate Fellow, The Ohio State University (1983).

1984-1985  NIGMS-MARC Predoctoral Fellowship, The Ohio State University.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS ................................................................. II
VITA ................................................................................................ v
LIST OF TABLES ............................................................................. x
LIST OF FIGURES .......................................................................... xli
LIST OF PLATES .............................................................................. xlii

CHAPTER PAGE
I. INTRODUCTION ........................................................................... 1
PURPOSE .......................................................................................... 1

GENERAL INTRODUCTION .................................................... 1
Chemical Mutagens and Point Mutations .................................. 2
The Relationship of Mutagenesis to Cancer ............................... 4
Point Mutations and Site Specific Mutagenesis ....................... 6
O-Alkyl Nucleotides and Mutagenesis ................................... 7
O4-Ethylthymine In Gap Misrepair Mutagenesis ..................... 8

DNA STRUCTURE AND SYNTHESIS IN MUTAGENESIS:
AN OVERVIEW ................................................................. 9
Structural Considerations ....................................................... 9
Mutagenic Physical Characteristics of Nucleotides ............... 14
The Role of DNA Polymerase In Base Selection .................... 17

RADIATION INDUCED MUTAGENESIS: AN INTRODUCTION .. 20
Environmental Agents and Mutagenesis ............................... 20
Ionizing Radiation and DNA Damage .................................. 21
DNA Damage From Ultraviolet Radiation .......................... 23
## CHEMICAL MUTAGENESIS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>General Considerations</td>
<td>25</td>
</tr>
<tr>
<td>Alkylation Agents</td>
<td>27</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Biological Importance of DNA</td>
<td>30</td>
</tr>
<tr>
<td>Alkylation</td>
<td></td>
</tr>
<tr>
<td>Mechanisms of Alkylation Damage Induced Mutagenesis</td>
<td>36</td>
</tr>
<tr>
<td>Site Specific Mutagenesis</td>
<td>37</td>
</tr>
<tr>
<td>Experimental Hypothesis</td>
<td>39</td>
</tr>
</tbody>
</table>

## II. MATERIALS AND METHODS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>MATERIALS</td>
<td>42</td>
</tr>
<tr>
<td>Reagents</td>
<td></td>
</tr>
<tr>
<td>Bacteriological Supplies</td>
<td>42</td>
</tr>
<tr>
<td>Nucleic Acid Enzymes</td>
<td>44</td>
</tr>
<tr>
<td>Chromatographic Materials</td>
<td>44</td>
</tr>
</tbody>
</table>

### SYNTHESIS OF O⁴-ETHYLTHYMIDINE TRIPHOSPHATE

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>General Protocol</td>
<td>45</td>
</tr>
<tr>
<td>Preparation of O⁴-Ethylthymidine with N-Ethyl, N-Nitrosoureas</td>
<td>45</td>
</tr>
<tr>
<td>Preparation of O⁴-Ethylthymidine via O⁴-Isopropyl Derivatization</td>
<td>47</td>
</tr>
<tr>
<td>Preparation of O⁴-Ethylthymidine Monophosphate</td>
<td>48</td>
</tr>
<tr>
<td>Reaction of O⁴-Ethylthymidine Monophosphate and Pyrophosphate</td>
<td>49</td>
</tr>
</tbody>
</table>

### MUTAGENESIS OF PUC8 BY GAP MISREPAIR

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutagenesis Misincorporation of O⁴-Ethylthymidine</td>
<td>51</td>
</tr>
<tr>
<td>Gap Misrepair Mutagenesis Protocol</td>
<td>51</td>
</tr>
<tr>
<td>Transformation of E. coli</td>
<td>60</td>
</tr>
<tr>
<td>Agarose Gel Electrophoresis</td>
<td>64</td>
</tr>
<tr>
<td>SalI Nicking Reaction</td>
<td>64</td>
</tr>
<tr>
<td>HinclII Nicking Reaction</td>
<td>65</td>
</tr>
<tr>
<td>Gapping Reaction</td>
<td>65</td>
</tr>
<tr>
<td>Misincorporation Reaction: The Effect of Polymerase Specificity</td>
<td>66</td>
</tr>
<tr>
<td>Nucleotide Pool Concentration Effects</td>
<td>66</td>
</tr>
<tr>
<td>E. coli Alkyltransferase Treatment</td>
<td>67</td>
</tr>
<tr>
<td>T₄ DNA Polymerase 3'-5' Misinsertion</td>
<td>67</td>
</tr>
<tr>
<td>Analysis of Mutants</td>
<td>68</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>The Relative Efficiencies of Different DNA Polymerases in O&lt;sup&gt;4&lt;/sup&gt;-Ethylthymidine Misincorporation Mutagenesis</td>
<td>78</td>
</tr>
<tr>
<td>2.</td>
<td>Effect of Pyrimidine Deoxyribonucleotide Pool Concentrations on O&lt;sup&gt;4&lt;/sup&gt;-EtTTP Misincorporation Mutagenesis</td>
<td>88</td>
</tr>
<tr>
<td>3.</td>
<td>The Effect of <em>E. coli</em> Alkyltransferase on the Mutation Frequency of Gap Misrepaired pUC8</td>
<td>92</td>
</tr>
<tr>
<td>4.</td>
<td>O&lt;sup&gt;4&lt;/sup&gt;-Ethylthymidine Triphosphate Misincorporation into <em>Hind</em>&lt;sup&gt;III&lt;/sup&gt; Nicked pUC8 Via T&lt;sub&gt;4&lt;/sub&gt; DNA Polymerase 3'-5' Exonuclease Exchange Reaction</td>
<td>94</td>
</tr>
<tr>
<td>5.</td>
<td>The Transformation Efficiencies for the Various Gap Misrepair Protocols</td>
<td>95</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURES</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. The four DNA bases; thymine, adenine, cytosine and guanine.</td>
<td>10</td>
</tr>
<tr>
<td>2. Thymine-adenine base pairing in a normal conformation and thymine-guanine base pairing with thymine in the ionized form.</td>
<td>16</td>
</tr>
<tr>
<td>3. Thymine-thymine dimer and thymine-cytosine (6-4) photoproduct.</td>
<td>24</td>
</tr>
<tr>
<td>4. The plasmid pUC8 containing the genes for beta-lactamase (ampicillin resistance) and beta-galactosidase (Lac Z').</td>
<td>43</td>
</tr>
<tr>
<td>5. The basic gap misrepair mutagenesis scheme of Hind.</td>
<td>57</td>
</tr>
<tr>
<td>6. The basic gap misrepair mutagenesis scheme of Sal.</td>
<td>58</td>
</tr>
<tr>
<td>7. The base pairing between O^4^-ethylthymine and adenine; and between O^4^-ethylthymine and guanine.</td>
<td>105</td>
</tr>
</tbody>
</table>
# LIST OF PLATES

<table>
<thead>
<tr>
<th>PLATES</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Titration of ethidium bromide concentrations in the nicking reaction of SalI.</td>
<td>53</td>
</tr>
<tr>
<td>II. Titration of ethidium bromide concentrations in the nicking reaction of SalI.</td>
<td>55</td>
</tr>
<tr>
<td>III. Randomly selected HincII mutants.</td>
<td>70</td>
</tr>
<tr>
<td>IV. HincII mutants obtained from gap misrepair by AMV DNA polymerase in the presence of OEtTTP as the sole pyrimidine substrate.</td>
<td>74</td>
</tr>
<tr>
<td>V. A comparison of PstI sensitive and resistant pUC8 isolates.</td>
<td>76</td>
</tr>
<tr>
<td>VI. Randomly chosen SalI resistant pUC8 isolates.</td>
<td>78</td>
</tr>
<tr>
<td>VII. Treatment of SalI resistant isolates with HincII.</td>
<td>81</td>
</tr>
<tr>
<td>VIII. Restriction endonuclease polymorphism of the shared SalI/TagI/HincII restriction endonuclease site of pUC8 generated by OEtTTP gap misrepair mutagenesis.</td>
<td>83</td>
</tr>
<tr>
<td>IX. Restriction endonuclease polymorphism of the shared SalI/TagI/HincII restriction endonuclease site of pUC8 generated by OEtTTP gap misrepair mutagenesis.</td>
<td>91</td>
</tr>
</tbody>
</table>
CHAPTER I
INTRODUCTION

PURPOSE

The mutagenicity of DNA base alkylation stems from the ability of these bases to interfere with the fidelity of DNA replication. Based on studies with various alkylated bases, it has previously been reported that they affect replication fidelity in a complex set of interactions which involve the structural and functional properties of DNA, DNA bases, polymerases, and repair systems. Although much work has been done to characterize the molecular aspects of the mutagenic potential of many alkylated bases, little has been done with O^-alkylthymines. O^-Alkylthymines are formed in small quantities following environmental or experimental exposure to alkylating agents such as nitrosoamines and nitosoureas. The modified base, O^-ethylthymine, which is poorly repaired in mammalian systems, may be a critically important base modification in the development of mutations and cancer.

The purpose of this study was to characterize and quantitate the mutagenic potential of O^-ethylthymine using the technique of gap misrepair mutagenesis. In a model in vivo replication system, incorporation of O^-ethylthymine into the SalI and HindIII restriction sites of the bacterial plasmid, pUC8, was predicted to
cause the generation of transition mutations at these sites. Transition mutants were then screened on the basis of their resistance to the endonuclease activity of the appropriate enzyme. The experimental results detailed here demonstrate that the O\textsuperscript{4}-ethylthymine is a potent mutagenic lesion. The mutagenic efficiency of this system was affected by the fidelity of the DNA polymerase, proofreading activity, the deoxynucleotide pool concentration, and repair by alkyltransferase.

**GENERAL INTRODUCTION**

Mutations arise from the generation of permanent, heritable alterations in genetic material. The relationship of DNA damage to mutagenesis is based on the ability of physical or chemical mutagens to interact with DNA and cause chemical modification of DNA components. Some mutagens react directly with DNA as a primary agent, others may require metabolic activation. Ultraviolet radiation and simple alkylating agents are good examples of direct acting mutagens (Singer, 1982; Haseltine, 1983). Ultraviolet light causes the formation of thymine glycol, pyrimidine dimers, and 6-4 photoproduct. Simple alkylating agents react with DNA via reactive electrophilic species, alkylazonium ion, for example (Magee, et al., 1976; Pegg, 1977). This interaction produces alkyl adducts at electron rich sites of DNA, primarily the oxygen and nitrogen positions. Mutagens which require metabolic activation include the arylamines, polycyclic aromatic hydrocarbons, aflatoxins, and nitrosoamines (Kriek and Westra, 1979; Miller, 1978; Magee, et al., 1973). Certain alkylating agents also have a therapeutic benefits as antineoplastic or
Immunosuppressive drugs. Cyclophosphamide, which requires metabolic activation, and the nitrosoureas; carmustine (BCNU), lomustine (CCNU), and semustine (methyl-CCNU), which do not require metabolic activation, are alkylating agents (Ludlum, 1977; 1978). The efficacy of these agents stems from their cytotoxic activity on tumor and myeloid cells at therapeutic doses. Ionizing radiations may damage DNA directly or indirectly by generating free radicals in the cellular environment which interact with chromatin and alter its integrity (Ward, 1981).

**Chemical Mutagens and Point Mutations**

Many chemical mutagens react with DNA and form covalent adducts (Singer, 1982). These adducts may alter nucleic acid structure in such a way as to interfere with the fidelity of the genetic code of the affected region during replication and give rise to point mutations (Drake and Baltz, 1976; Singer and Kusmierek, 1982). Point mutations result from the substitution of a different nucleotide base at one site. The biological significance of a point mutation depends on the ultimate effect it has on the structure or expression of the affected gene. However, quite efficient DNA repair processes have evolved to overcome some of the mutagenic consequences of DNA damage. Nevertheless, some alterations do escape these surveillance systems or are not repaired and yield mutational effects. The generation of point mutations is interesting because of their implication in the activation of oncogenes (Reddy, et al., 1982). Point mutations have also been associated with other disorders such as beta-thalassemia.
The mechanisms by which point mutations arise may be due in part to the ability of modified DNA bases to cause misincorporations during DNA synthesis (Topal and Fresco, 1976).

The Relationship of Mutagenesis to Cancer

The relationship between mutagenesis and carcinogenesis is more complex and not altogether simply defined by the process of nucleic acid alterations. There is, however, a relationship between carcinogenesis and mutagenesis based on epidemiologic observations which associate environmental agents with the incidence of human cancer (Shubik, et al., 1984). For example, diet and colon cancer (Wynder, et al., 1983), aflatoxins and liver cancer (Sun, et al., 1983), cigarette smoking and bronchogenic carcinoma (Hoffman, et al., 1983), and radiation induced leukemias (Caldwell, et al., 1980) are all well established examples of the epidemiologic relationship of environmental mutagenesis to carcinogenesis. Whole animal and tissue culture models have also been developed to assay the mutagenic activity of many potential carcinogens (Bogovski and Bogovski, 1981; Brown, et al., 1983).

Another method which has been developed to characterize the relationship between mutagenesis and carcinogenesis is the Ames assay (McCann, et al., 1975). However, the association of mutagenic DNA damage with carcinogenesis is at present correlative and not thoroughly defined, explained or understood. The process of carcinogenesis involves other changes in biological mechanisms which
regulate cellular differentiation, and govern the structure and function of the cell. Just how these regulatory mechanisms relate to the mutagenic process of carcinogenesis is unclear (Borek, 1984).

Recent advances in molecular biology have shown that in certain cancer cell lines there are regulatory genes which contain point mutations not found in normal cells derived from the same tissue. For example, a point mutation in the H-ras gene isolated from a human bladder carcinoma cell line has been shown to correlate with transforming activity in vitro (Taparowsky, et al., 1982). When this mutated gene was transfected into NIH 3T3 murine fibroblasts, the fibroblasts which expressed this gene acquired the in vitro characteristics of neoplastically transformed cells: loss of contact inhibition, and anchorage independent growth in soft agar. Similar results were demonstrated with the H-ras isolates from MNU induced rat mammary carcinomas (Sukumar, et al., 1983). The ras oncogene is one of several oncogenes described in the literature (Slamon, 1984). These genes appear to be important regulatory genes, which in tumor cells are quite often altered (Heldin and Westermark, 1984). Another interesting aspect of oncogenes is their association with the transforming property of many acute transforming retroviruses (Spector, et al., 1978; Dhar, et al., 1982). Thus, it appears that DNA damage induced carcinogenesis may be related to alterations in the functionality and integrity of certain genes which govern cellular differentiation and function.
Point Mutations and Site Specific Mutagenesis

The biological importance of small mutagenic lesions in DNA has been shown to have relevance to disease states in man and animals. However, the role of mutagenesis has further application to the study of nucleic acid structure, function and enzymology. Several methods of site specific mutagenesis have been developed to study the effects of localized mutagenic lesions on functional genes; rabbit beta-globin gene (Muller, et al., 1978) and Simian Virus 40 large T antigen (Kalderon and Smith, 1984), for example. Similarly, the techniques of site specific mutagenesis are being applied to manipulate the primary structure of proteins (Dalbadie-McFarland, et al., 1982) and enzymes (Winter, et al., 1982; Villafranca, et al., 1983) and to observe how posttranslational modification of these peptide chains alters the final protein structurally and functionally (Carter, et al., 1984; Straus, et al., 1985).

In several studies site specific mutagenic techniques have been applied to the study of the mutagenic effects of modified nucleotides such as N3-hydroxycytosine (Domingo, et al., 1976) and O6-methyldeoxyguanosine (Eadle, et al., 1984). The strength of such studies lie in the simple and elegant way in which one may assay the effect of base modifications in nucleic acids to ascertain the functional and heritable consequences of such changes. Such systems once established may eventually find relevance in the study of larger and more complex eucaryotic systems.
O-Alkyl Nucleotides and Mutagenesis

O\textsuperscript{6}-Methyldeoxyguanosine is a potently mutagenic alkynucleotide formed by alkylating carcinogens such as N-methyl-N-nitrosourea (MNU) (Pegg, 1977; Singer, 1979). Repair of this alkyl modification in mammalian and some bacterial systems involves a methyltransferase which removes the O\textsuperscript{6}-alkyl group and covalently binds it to a cysteine residue (Scicchitano and Pegg, 1982). The persistence of O\textsuperscript{6}-methyldeoxyguanosine in DNA has been characterized as a potent mutagenic lesion associated with a high incidence of tumorigenesis in animal models (Goth and Rajewsky, 1974; Singer, 1979). This alkylated nucleotide has been demonstrated to generate point mutations such as G-C to A-T transitions and G-C to T-A transversions in DNA synthesis in vitro (Dodson, et al., 1982; Toorchen and Topal, 1983) and DNA replication of bacteriophage templates in vivo (Eadle, et al., 1984).

Other O-alkynucleotides are produced by alkylating agents; O\textsuperscript{2}-alkyldeoxycytosine, O\textsuperscript{2}- and O\textsuperscript{4}-alkylthymine (Singer and Kusmlerek, 1982). Of these, O\textsuperscript{4}-alkylthymine may have the greatest ability to cause error prone DNA synthesis (Brennand, Saffhill, and Fox, 1982). Previous studies have shown that O\textsuperscript{4}-ethyl- and O\textsuperscript{4}-methylthymine may misincorporate against guanine residues during in vitro transcription of oligonucleotide substrates (Singer, Sagl, and Kusmlerek, 1983). O\textsuperscript{4}-Ethylthymine is a persistent DNA lesion in many systems. It is not as readily repaired as O\textsuperscript{6}-methylguanine (Bodell, et al., 1979; McCarthy, et al., 1983; Pegg, et al., 1983; Ahmmed and Laval, 1984; Dolan, et al., 1984).
Based on the information currently known about the mutagenicity of \( O^4 \)-ethylthymine, it may be hypothesized that it is a potent mutagenic DNA lesion resulting in the generation of point mutations by causing misincorporation of noncomplementary bases during DNA synthesis which generates point mutations. Because of the persistence of this lesion, the mutagenic incidence of \( O^4 \)-ethylthymine induced lesions may be higher in proportion to its frequency of occurrence than previously believed. By using the technique of gap directed misincorporation, the objective of this study is to characterize the mutagenic efficiency of \( O^4 \)-ethylthymine in a bacterial plasmid, PUC8. Essentially, when incorporated into a restriction site such as \textit{SalI} or \textit{HinclI}, \( O^4 \)-ethylthymine will yield transition mutations which will abolish the ability of these restriction enzymes to cleave the plasmid at this site, an easily detectable mutational change.

In the development of this hypothesis and experimental system, it will first be necessary to evaluate the background of DNA damage and repair: the properties of the chemical carcinogens, alkylating agents in particular; the properties of DNA replication and fidelity of DNA replication; and the theoretical background of site specific mutagenesis.
DNA STRUCTURE AND SYNTHESIS IN MUTAGENESIS: AN OVERVIEW

Structural Considerations

Deoxyribonucleic acid (DNA) is the predominant repository of genetic information in living systems. It has an infrastructure composed of alternating phosphate/2'-deoxyribose units linked via phosphodiester bonds at the 5' and 3' positions of the sugar moiety. A nucleotide base is attached to the C-1 position of deoxyribose through an N-glycosidic bond. There are four physiologic bases; the purines adenine (A) and guanine (G), and the pyrimidines cytosine (C) and thymine (T) (Figure 1). In the physiologic state, DNA is composed of two antiparallel strands in a double helical configuration 20 Å in diameter. The helical form has a deep (major), and a shallow (minor) groove; the base pairs are stacked one over the other in parallel planes which are perpendicular to the axis of the helix and to the plane of the deoxyribose. Nucleotide bases form complementary purine-pyrimidine pairs which hold the two DNA strands together by hydrophobic interaction, and by hydrogen bonding. The hydrogen bonding of complementary base pairs is specific: A-T and G-C pairs are thermodynamically and enzymatically favored (Figure 1).

DNA Replication. The function of DNA as an information storage medium is based upon the sequence the nucleotide bases. These sequences may either be functional in the sense that they contain the design of a particular peptide, or they may be regulatory in nature, containing information regarding the expression of a gene or genes. During DNA replication the DNA polymerase enzyme reproduces the
Figure 1. The four DNA bases; thymine, adenine, cytosine, and guanine. Hydrogen bonding between the thymine and adenine, cytosine and guanine is illustrated.
nucleic acid sequence by producing a strand of bases complementary to the template parental strand. DNA synthesis is semiconservative; each replication yields two new molecules having one parental strand and one newly synthesized daughter strand. Semiconservative replication by nature helps to maintain the integrity of the genetic code.

Transcription and Translation. DNA is transcribed via RNA polymerases into a ribonucleic acid (RNA) transcript: a single strand composed of bases complementary to the DNA template of the gene. The transcript, messenger RNA (m-RNA) is interpreted as a set of base triplets, codons, each specifying an amino acid. These codons are translated in the ribosomal framework of the cell by transfer RNA (t-RNA) molecules. T-RNA is composed of an anticodon loop which is complementary to a codon triplet in the RNA transcript and a 3'-terminus to which an amino acid is bound. Thus, during translation, the genetic code is interpreted by the sequential annealing of t-RNAs to the codons of an m-RNA transcript to yield their 3'-amino acyl termini to the growing peptide chain. The primary peptide may be posttranslationally modified to yield the final protein product.

The Importance of Sequence Fidelity. Base sequence in nucleic acids is of ultimate importance at all levels since the structural properties of a gene product depend upon faithful transcription and translation of the gene. Even more critical, however, is the faithful maintenance of the DNA sequence itself since it is this information that is passed on to later generations of cells (Loeb and Kunkel, 1982).
Spontaneous Mutagenic Events

Spontaneous hydrolysis occurs in nucleic acids under physiologic conditions leading to deaminations (Lindahl and Nyberg, 1974; Karran and Lindahl, 1980), depurinations (Lindahl and Nyberg, 1972), and depyrimidations (Schapper, et al., 1982).

Spontaneous Deaminations. For instance, cytosine and adenine spontaneously deaminate to yield uracil and hypoxanthine residues, respectively (Lindahl and Nyberg, 1974; Karran and Lindahl, 1980). The spontaneous deamination of cytosine is favored thermodynamically and is a more frequent occurrence than the spontaneous deamination of adenine. Furthermore, single stranded DNA is more susceptible to spontaneous deamination events by two orders of magnitude (Lindahl, 1979). Unrepaired deamination is a directly mutagenic event which can lead to the formation of a novel base pair during replication which alters the sequence at that point (Duncan and Weiss, 1978).

Spontaneous Hydrolysis of the N-glycosidic Bond. Hydrolysis of the N-glycosidic bond to yield an abasic site is more frequent at purinic sites than at pyrimidinic sites, and occurs with a greater frequency in single stranded DNA than in double stranded DNA (Lindahl and Nyberg, 1972; Lindahl, 1979; Hartman, 1980). In fact, depurination has been estimated to occur at a rate of $10^4$ random events per cell per day in mammalian systems (Lindahl and Nyberg, 1972). Abasic sites may result in strand scission by the hydrolysis of the phosphodiester bond at that point (Lindahl and Andersson,
1972). If not, the absence of template information at this point may interfere with the fidelity of replication (Loeb and Kunkel, 1982). Abasic sites may prove to be lethal or cause base substitution errors (Kunkel, 1984).

**Effects of Apurinic/Apyrimidinic Sites on DNA Synthesis.** Error prone synthesis at apurinic/apyrimidinic (AP) sites may be demonstrated by *in vitro* polynucleotide template transcription (Bolteux and Laval, 1982) with DNA polymerases, and *in vivo* when the SOS response in *Escherichia coli* has been induced (Schaaper and Loeb, 1981).

Previous studies have shown that base substitution across from abasic sites strongly favors the insertion of adenine (Schapper, Kunkel, and Loeb, 1983). For example, *in vitro* reversion frequencies of amber mutations in bacteriophage ØX174 DNA containing abasic sites indicate a definite preference for adenine insertion in these sites. The basis for this phenomenon appears to be inherent properties of the AP site and the replicative framework (Loeb and Kunkel, 1982). Similarly, adenine was found to be preferentially inserted at AP sites in depurinated M13mp2 phage transfected into SOS induced *E. coli* (Kunkel, 1984).

**Repair of Spontaneous Mutagenic Lesions.** Because of the frequency of spontaneous deaminations, depurinations, and depyrimidinations in DNA, repair systems to circumvent the potential lethal or mutagenic effects of these lesions are necessary. In the case of deaminated cytosine and adenine residues, an excision-repair system has been characterized (Lindahl, 1982). The first step
Involves the removal of the deaminated base by the enzymatic hydrolysis of the N-glycosidic bond to create an abasic site. Subsequently, the abasic site is excised by an apurinic/apyrimidinic endonuclease, creating a gap. The gap is filled in and ligated using the complementary strand as a template.

Glycosylases specific for the repair of uracil and hypoxanthine residues have been found in bacterial and mammalian systems; Uracil-DNA glycosylase and Hypoxanthine-DNA glycosylase (Karran and Lindahl, 1978; Caradonna, et al., 1980; Krokan and Wittwer, 1981; Lindahl, et al., 1977).

The repair of abasic sites in DNA is predominantly carried out by apurinic/apyrimidinic endonucleases. These enzymes produce 5'-phosphate and a 3'hydroxy termini which serve as substrates for DNA polymerase to initiate repair synthesis of the affected region using the complementary strand as a template (Lindahl, 1982). The apurinic/apyrimidinic endonucleases recognize abasic sites and excize the phosphodeoxyribose at these sites.

Mutagenic Physical Characteristics of Nucleotides

Rare Base Conformations. Molecular characteristics of nucleosides such as ionization, tautomerization, and anti-syn rotation about the glycosyl bond may produce spontaneous mismatches during DNA synthesis. Rare base conformations occur randomly and are reversible. The equilibrium constants for bases in tautomeric shifts have been calculated at $10^4$ mole$^{-1}$ sec$^{-1}$ (Topal and Fresco, 1976); the pK values for ionized bases in single stranded DNA are on the order of $10^{-2}$ to $10^{-5}$ mole$^{-1}$ sec$^{-1}$ (Singer, 1975); rotameric forms have
a predicted $K_{eq}$ of $10^{-1}$ to $10^{-2}$ mole$^{-1}$sec$^{-1}$. This material is detailed in a review by Singer and Kusmierek (Singer and Kusmierek, 1982).

Ionization of bases may allow for the generation of anomalous base pairs by allowing hydrogen bonding to noncomplementary bases (Figure 2.). Watson and Crick accounted for spontaneous transition mutations by first postulating a purine-pyrimidine mispairing scheme in which one base in a rare imino or enol tautomeric form could mispair with a normally noncomplementary nucleoside (Watson and Crick, 1953). These forms allow stable hydrogen bonding to occur between noncomplementary bases. Experimental evidence has confirmed this hypothesis (Drake and Baltz, 1976; Fresco et al., 1980) which has been expanded to account for purine transversions (Topal and Fresco, 1976). In this model, the template base is in a rare tautomer state and the incoming purine dNTP is in the syn rotatory conformation.

**The Passive Polymerase Model.** Observations for base pairing frequencies between the base analog 2-aminopurine, thymine and cytosine to yield 2-AP-C and 2-AP-T mispairs conflict with rare tautomer model predictions (Watanabe and Goodman, 1981). Consequently, another model has been proposed to account for base mismatch on the grounds of free energy hydrogen bonds between bases. In such a model system, DNA polymerase plays a passive role in DNA synthesis based on differences in the free energies of base pairs (Goodman, et al., 1982). Excluding the influence of enzyme
Figure 2. Thymine-adenine base pairing in a normal conformation and thymine-guanine base pairing with thymine in the ionized form.
specificities during nucleotide synthesis, the frequency of misincorporations may be predicted mathematically on the basis of their free energies of association alone:

\[ \text{Equation 1.} \quad -G = RT \ln (\text{Correct/Incorrect}) \]

(Loeb and Kunkel, 1982). DNA polymerases in this depiction function only to polymerize deoxyribonucleotide substrates along a template DNA strand. The nucleotide complementation specificities are determined by hydrogen bonding free energies.

Pyrimidine-purine transversions are not theoretically feasible on the basis of tautomerism, N-glycosyl rotation, or ionization, but may be the result of stacking forces (Topal and Fresco, 1976; Singer and Kusmlerek, 1982).

These base pairing models rely on the physical properties of the nucleosides which allow for mismatch complementation; the physical properties of geometrical configuration and the free energy of hydrogen bonding forces between matched and mismatched bases which conform to the torsional constraints of the double helix.

The Role of DNA Polymerase In Base Selection

Contradictions of the Passive Polymerase Model. Other than the molecular characteristics of the nucleic acid bases themselves, enzymatic factors also play an important role in the fidelity of DNA synthesis. Measurements of spontaneous mutation frequencies indicate that spontaneous base pair substitutions occur between $10^{-7}$ to $10^{-11}$ misincorporations per base pair replicated (Cox, 1976; Coulondre and Miller, 1977). An error rate of $10^{-4}$ to $10^{-6}$ has been reported for \textit{in vitro} synthesis of polydeoxynucleotides by \textit{E.}
coll DNA polymerase I (Agarwal, 1979). The error frequency appears to be lower when natural DNA substrates are used as templates for In vitro DNA synthesis. The forward mutation rates of various organisms appears to range in orders of magnitude between $2 \times 10^{-8}$ for DNA phages to $7 \times 10^{-11}$ for Drosophila per base pair replicated (Drake, 1976).

The equation:

\[
(Equation \ 1.) \ \Delta G = RT \ln \frac{\text{Incorrect}}{\text{Correct}}
\]

would predict an error frequency of 1 mispaired nucleotide per 10-100 nucleotides incorporated, a rate confirmed by the nonenzymatic polymerization of synthetic oligopolyribonucleotides. However, the observations just noted are much lower than the predicted mutational frequencies expected by this equation. Thus, the fidelity of DNA polymerases must be influenced not only by the physico-chemical and hydrogen bonding properties of DNA bases themselves, but are also affected to a large extent by the specificities of the enzyme as well.

T4 DNA Polymerase Base Specificities. Studies of the bacteriophage T4 provided the initial evidence for DNA polymerase specificities during DNA synthesis (Loeb and Kunkel, 1982). Mutants of the DNA polymerase gene, gene 43, of T4 bacteriophage produce a wide variety of increased and decreased point mutation rates compared to wild type (Ripley, 1981). Mutations of this gene also produce variants of frame fidelity: frameshift inducer and frameshift suppressor polymerases (Ripley and Shoemaker, 1982).
DNA Polymerase Selectivity at Abasic Sites. Furthermore, the preferential insertion of adenine into M13mp8 phage apurinic sites during SOS replication supports the premise that DNA polymerases act in a selective manner (Kunkel, 1984). Even when AMV DNA polymerase, a nonproofreading enzyme, is used for in vitro synthesis of M174 DNA, the mutational reversion frequencies for amber mutants in this bacteriophage are far below the predicted values based on the free hydrogen bonding energy relationship (Loeb and Kunkel, 1982). Thus, the DNA polymerase enzymes also show a substrate specificity independent of the influence of hydrogen bonding, proofreading activity, or base conformations.

The Role of Polymerase Proofreading Activity. The 3'→5' exonuclease activity of several DNA polymerases is a proofreading function that edits the process of 5'→3' strand synthesis in order to prevent base misincorporation. DNA polymerases isolated from bacteria and bacteriophages have this function (Setlow, Brutlag, and Kornberg, 1972; Muzyczka, 1972). The characterization of this activity in eucaryotic systems is not well defined. Purified DNA polymerase preparations from many eucaryotic sources fail to demonstrate 3'→5' exonuclease activity (Kunkel, Tcheng, and Myer, 1978; Loeb and Kunkel, 1982). The DNA polymerase from Avian Myeloblastosis Virus totally lacks proofreading capability (Battula and Loeb, 1974; Bernardi and Ninio, 1978). However, other investigators have characterized this function in some sources (Byrnes, et al., 1976; Chen, et al., 1979; Mosbaugh and Meyer, 1980).
Summary of Mechanisms in Spontaneous Mutagenesis. Given what has been described pertaining to the fidelity of DNA synthesis, it is regulated at four distinguishable levels: the physicochemical properties of the nucleoside bases and their ability to pair in a manner conforming to the geometry of the DNA double helix; the free energy of the hydrogen bonds between DNA bases, and the passive role of polymerases in this process; enzyme specificities regarding complementarity and frameshift integrity in substrate and template; and 3'-5' exonuclease proofreading specificities which vary between sources and enzyme types. In turn, spontaneous mutations may originate as the result of random thermodynamic instabilities of DNA bonds; from the properties of base conformations and hydrogen bonding energies; or may result from the specificities and proofreading functions of the DNA polymerase enzymes.

RADIATION INDUCED MUTAGENESIS: AN INTRODUCTION

Environmental Agents and Mutagenesis

Physical and chemical agents in the cellular environment have the capacity to interact with chromatin material and generate mutagenic DNA damage. The various environmentally induced forms of DNA damage include base substitutions and mismatches, strand breaks, abasic sites, strand crosslinking, covalent binding to cellular macromolecules, intercalation, and aryl and alkyl adduct formation. The major damage inducing agents discussed here include; ionizing radiation, ultraviolet light, heat, therapeutic and experimental nucleotide analogs, electrophilic aryl and alkyl compounds. The relevance of these factors to the development of the theoretical
background of mutagenesis is important and a brief review of the
mechanisms leading to the understanding of environmental genotoxins is
warranted here.

**Ionizing Radiation and DNA Damage**

Ionizing radiation includes the high energy spectrum of
electromagnetic radiation, i.e. X- and gamma rays, as well as
particles, for example, alpha, beta, and neutrons. The lethality and
mutagenic characteristics of these agents are well known and have been
extensively reviewed (Prashad, 1984; Hall, 1982). However, the
various mechanisms underlying their mutagenic activities are still
being characterised.

Ionizing radiation induces a variety of lesions in the genetic
material of the cell: single strand breaks (Hutchinson, 1978), double
strand breaks, sugar and base alterations (Ward and Kuo, 1973; Skov,
Palcic, and Skarsgard, 1982), and binding to macromolecules (Olinsky,
Briggs, and Hnilica, 1981) are known to occur. High energy
interactions between these radiations and the molecular environment of
the cell results in the generation of ions, peroxides and free
radicals (Ward, 1981). The predominant molar concentration of water
in the cell generates the majority of reactive oxidative species which
cause the most intracellular damage (Greenstock, 1981). Hydroxyl
radical (OH·), and superoxide ion O₂⁻ appear to be the major active
species leading to the production of chromosomal alterations (Sasaki
and Matsubara, 1979; Mittler, 1981), cell death (Johansen and
Howard-Flanders, 1965; Sanner and Pihl, 1969; Skov, 1984), and DNA
Other than the specific interaction of the chromatin material with ionizing radiation directly or via reactive oxidizing species, gamma radiation also suppresses the activity of DNA polymerase (Leadon and Ward, 1981).

It appears that strand breakage, and gross chromatin alterations are the most biologically significant effects of ionizing radiation. The repair of this sort of damage involves a complex of activities. Nuclease activity specific for alkali labile and alkali stable radiation damage in DNA has been characterized for E. coli (Strniste and Wallace, 1975; Wallace, Armel, and Katcher, 1978). Mammalian systems for the rejoining of DNA strand breaks have been described (Lett, Keng and Sun, 1978; Hutchinson, 1978, 1979; Curry and Cole, 1973). Although the molecular mechanisms of such repair are still not thoroughly elucidated, these processes are believed to involve DNA polymerases and ligases in various strand joining, excision repair, repair synthesis, and crossover repair synthesis models. The enzymes involved in strand break repair processes also repair nicks and abasic sites generated by the free effects of ionizing radiation and are demonstrably saturable (Dugle, Gillespie, and Chapman, 1976; Ward, 1981). Hence, the cytotoxic and mutagenic events associated with ionizing radiation may reflect the substrate saturation of the repair enzymes, erroneous rejoining of doubly cleaved DNA, and the inhibition of polymerase activity by radiation, as well as mismatch repair, strand insertions and deletions.
DNA Damage From Ultraviolet Radiation

The biological consequences of ultraviolet light exposure include lethality, virus induction, mutagenesis, and tumorigenesis (Haseltine, et al., 1982). DNA absorbs light in the frequencies between 286 and 300 nm (Haseltine, 1983). There are various DNA lesions associated with this interaction including pyrimidine cyclobutane dimers (Wang, 1976), and 4-6 photoproduct (Lippke, et al., 1981) (Figure 3.). The production of pyrimidine dimers predominates in UV induced damage and appears to account for the large share of the biological consequences of UV light exposure. Cyclobutane pyrimidine dimers distort the helical configuration of the DNA molecule in their proximity. This distortion interferes with DNA synthesis and may terminate this process (Moore, et al., 1981). Thus, pyrimidine dimers are inherently mutagenic. The removal of these dimers is catalyzed by repair enzymes (Seeburg, 1981; Sancar and Rupert, 1978) or spontaneous photoreversal by 360 to 500 nm light (Sutherland, 1978). Various repair deficient mutants have been characterized. A classic example in man is the hereditary complex of genetic disorders, Xeroderma pigmentosum. This disorder predisposes those affected to the immediately deleterious effects of UV damage and is related to defective DNA repair systems for UV induced DNA damage (Friedberg, et al., 1981).

Reapir of Radiation Damage. The repair of such damages involves an excision-repair scheme. The dimer is recognized by a specific endonuclease which creates a single strand nick 5' to the lesion. The 5'-3' exonuclease activity of DNA polymerase creates a gap from this
Figure 3. Thymine-thymine dimer and thymine-cytosine (6-4) photoproduct.
nick which is filled in by the 5'-3' polymerase activity of DNA polymerase. DNA ligase closes the nick generated by this process, and the DNA is returned to its original state. Recent modifications of this scheme have been put forth, all of them are based on the excision-repair format (Lindahl, 1982). The presence of a pyrimidine dimer glycosylase has been suggested in some systems, yet this activity does not appear to prevail in most cases (Haseltine, et al., 1980; Gordon and Haseltine, 1981).

Recent evidence has led to the proposal that the 6-4 photoproduct is quite mutagenic to DNA and is not repaired as efficiently as the cyclobutane dimers (Brash, and Haseltine, 1982). According to this report, the specificity of UV induced mutagenesis involving cyclobutane dimers and 4-6 photoproduct lies within pyrimidine rich regions.

CHEMICAL MUTAGENESIS

General Considerations

Chemical agents act as mutagens by interacting with DNA in such a way as to modify the structure or sequence of the affected region. The formation of covalent adducts and the hydrolysis of bonds account for most of the mutagenic effects.

Adduct formation results from the electrophilic addition of a reactive species with electron rich areas of the DNA strand. While some chemicals require metabolic activation to become mutagenic, others may exert their mutagenic activity directly.
**Metabolic Activation.** Metabolic activation of mutagens has been the subject of intense investigation for many years now. Aromatic amines and polycyclic hydrocarbons are enzymatically modified in the complex microsomal environment of mammalian cells to form mutagenic metabolites which damage DNA, a step which is essential to the development of their mutagenic activity (Miller and Miller, 1981). Important examples of this class of carcinogens are; benzo[a]pyrene (B[a]P), acetylaminofluorene (AAF), aflatoxin B₁ and 7,12 dimethylbenz[a]anthracene.

The arylamines include 2-AAF, N-hydroxy-2-naphthylamine, N,N-dimethyl-4-aminobenzene and related compounds. The mutagenic activation of these compounds in the liver involves enzymatic conversion to sulfate ester and N-acetoxy derivatives by sulfotransferase and N,O-acetyltransferase enzymes, respectively (Debaun, Miller and Miller, 1970, King, 1974). Deacetylase activity converts the N-acetyl derivative to the ultimate N-hydroxy reactive species. The sulfated compound is quite electrophilic and readily reacts with DNA (Grunberger, 1983). However, the role of free radical conversion of these compounds in the liver and other organs is also a plausible route of activation and is supported by much experimental evidence (Floyd, 1981). Adduct formation at the N² and C-8, O⁶-positions of guanine and the N⁶-position of adenine have been characterized (Singer and Kusmlerek, 1982).

The metabolic activation of PAH is known to involve microsomal enzymes (Yang, Deutsch, and Gelboin, 1978; Bartsch, et al., 1980). Adduct formation has been described at N²-G, N⁶-A, N⁷-G by
B[a]P (Jeffrey, et al., 1976, 1979). The formation of a PAH adduct is a bulky lesion which can inhibit DNA synthesis, cause strand breaks, or generate apurinic sites, especially by forming N\textsuperscript{7}-G, and N\textsuperscript{3}-A adducts. The major route of bulky adduct repair is similar in many respects to the excision-repair systems characterized in bacteria, mammals, and humans (Lindahl, 1982).

**Alkylating Agents**

**General Considerations.** The main issue of this investigation involves characterizing mutagenicity of the minor alkylaion product in DNA, O\textsuperscript{4}-ethylthymine. This alkylated base is produced by the action of alkylating agents on DNA.

A large share of mutagens are simple alkylating agents. The extent of reactivity and mutagenicity to DNA varies between compounds. It may be generally stated that N-nitroso agents are more mutagenic than alkyl sulfates, and that methyaling agents are more reactive than are ethylating agents, and higher carbon alkyl derivatives (Singer and Kusmierek, 1982). The nitrogen and oxygen residues in nucleic acids and oligonucleotides are quite electron rich and susceptible to alkylation in aqueous solution at neutral pH. The exocyclic amines and N-1 of guanine are not found alkylated in vivo. This aspect of the chemistry of these compounds which is markedly different from that of the larger polycyclic hydrocarbons and the aryl amines.

**Reactivities of Alkylating Agents.** Alkyl sulfates, tend to react with nitrogenous substituents, and N-nitroso compounds react mostly with oxygen residues (Singer, 1982). In fact, ethylating
agents are more selectively reactive towards oxygen residues than are their methylating analogs (Singer, et al., 1978). The ethyl substitutions of DNA by the N-ethyl, N-nitroso compounds; N-ethyl-N-nitrosourea, or N-methyl-N'-ethylnitrosoguanidine, occur at the following sites; phosphate>> N-7 G> O2T> O6G> N3-A> O4T, O2-C> other substituents (Singer and Kusmlerek, 1982). In vivo, 80% of the reaction products of N-ethyl-N-nitrosourea with DNA are on oxygen. Ethylphosphotriesters are the predominant product (50-60%), followed by O6-ethylguanine and O2-ethylythymine (7-9%), O4-ethylythymine and O2-ethylcytidine (2-4%). The 2' oxygen of ribose is also alkylated in RNA. The high degree of oxygen specificity of these reagents correlates well with their mutagenicity. For example, ENU and ENNG are more oxygen reactive and mutagenic than MNU and MNNG which in turn are more so than EMS, MMS and diethylsulfate, respectively (Singer, 1982).

The reactivity of simple alkylating agents has been assayed on single stranded ribonucleotides, deoxyribonucleotides and double stranded DNA. There are differences in the reactivities of these chemicals at the N-3 of cytidine and the N-1 of adenine between single and double stranded DNA molecules. Apparently, the differences in these reactivities is related to the hydrogen bonding activities of these nitrogen loci in the double stranded molecule, hence, the single stranded molecule is more reactive at these positions. Strand denaturation at physiologic temperatures, 37°C is enough to allow some reactivity in double stranded polynucleotides. The extent of alkylation of nucleoside oxygens is not a function of strandedness,
however. The O\textsuperscript{6}-guanine, and O\textsuperscript{2-,} O\textsuperscript{4}-thymine sites have unpaired electrons, and the O\textsuperscript{2-}thymine site is not involved in hydrogen bonding (Singer, 1982; Jensen and Reed, 1978).

Another class of mutagenic and carcinogenic alkylating compounds are the N-alkyl-N-nitrosoamines which are also highly oxygen reactive and are exemplified by the compounds diethyl-\textsuperscript{-}, and dimethyl-nitrosoamine. Metabolic activation is necessary for the nitrosoamines to bind to DNA (Pegg, 1977). Biologically reactive nitrosoamine derivatives may be biosynthesized \textit{in vivo} from nitrite or secondary and tertiary amines (Bartsch, et al., 1983).

The reaction mechanism for the alklylation of oxygen and nitrogen substituents in nucleotides by the alkynitroso compounds involves enzymatic or spontaneous dissociation to an electrophillic alkylidiazonium cation which binds to electron rich nitrogen and oxygen sites in DNA (Pegg, 1977).

\textbf{Cyclic Alkylating Agents}. Two other important classes of alkylating mutagens include the cyclic alkylating agents and the halonitrosoureas. The cyclic alkylating agents are unstable reactive ring structures: epoxides (chloroethylene oxide), lactones (propiolactone), S-mustards (mustard gas), and N-mustards (HN-2). These compounds form a variety of monoadducts primarily at the N-7 position of guanine, and to a lesser extent at the N-1 of adenine and N-3 of cytidine. Bifunctional S-mustards and N-mustards form crosslinks via N-7 guanine residues. The mutagenic derivatives of vinyl chloride, chloroethylene oxide and chloroacetaldehyde are known carcinogens in man. These mutagenic effects derive from their
propensity to form fluorescent cyclic etheno derivatives of the exocyclic nitrogens in adenine, guanine, and cytosine: 1,N⁶-ethenoadenine, 1,N²-ethenoguanine, and 1,N⁴-ethenocytidine. The halonitrosoureas include antineoplastic agents and mutagens. These are predominantly bifunctional compounds capable of forming crosslinks or cyclic ethano derivatives (Sattsangl et al., 1977; Singer and Kusmlerek, 1982; Kusmlerek and Singer, 1982; Lab, 1982).

The Biological Importance of DNA Alkylation

The significance of DNA alkylation in regard to carcinogenesis has long been investigated. Alkylation of the N-7 residue of guanine was originally believed to be related to the development of cancer and mutagenesis. The low mutagenic efficiency of the methylalkyl sulfonates was in contrast to their high levels of N-7 alkylation, clearly minimizing the importance of this lesion in mutational potency. However, the production of O⁶-alkylguanine residues by ethylmethane sulfonate, ethylnitrosourea and methylnitrosourea correlated quite highly with increased mutagenicity (Frei, et al., 1978; Singer, 1982).

The proposition that O⁶-alkylguanine residues were fundamental mutagenic lesions was first promulgated in the light of evidence which demonstrated the generation of point mutations in T-even bacteriophages corresponded to the relative differences in O⁶-alkylguanine production by MMS and EMS; the latter being mutagenic and an O-alkylator, the former being neither (Loveless and Hampton, 1969). The presence of O⁶-methylguanine was demonstrated in E. coli (Lawley and Orr, 1970), and MNNG treated cell cultures
(Lawley and Thatcher, 1970), and subsequently was associated with tumorigenesis in animal models (O'Connor, 1973). As may be recalled, methylmethane sulfonate, a potent N-alkylating agent, but a weak O-alkylator, is weakly mutagenic. In contrast, N-methyl, N-nitrosourea is a weak N-alkylator, strong O-alkylator and is highly mutagenic (Loveless, 1969).

**Relationship of Alkylation Damage to Carcinogenesis.** The mutagenic activity of alkylating compounds also correlates with their carcinogenic potential. For example, MNU, a potent hepatocarcinogen, produces substantially higher levels of O6-alkylguanine residues in rat liver compared to the relatively noncarcinogenic MMS (O'Connor, 1973). The correlation of O6-alkylguanine base lesions to tumorigenesis is also influenced by organ and tissue specificities and by the persistence of this lesion in affected tissues. Exposure to alkylating agents and the distribution of alkylation damage to DNA in the tissues varies among different agents and hence determines to some extent the types of cancers produced. There are also species differences in the tumorigenicity of these substances. These differences reflect the pharmacokinetics, bioavailability, dissociation and enzymatic activation and removal at various levels. The nitrosoureas ionize at physiological pH and temperature and hence the level of alkylation in the tissues represents the distribution of these compounds following intravenous administration (Pegg, 1977, 1980; Montesano, 1981).
The Importance of DNA Repair. Perhaps the most important factor regarding the genotoxicity of $O^6$-alkylguanine, and indeed, the host of other $O$-alkylated nucleotides, is the ability of the cell to repair these damages. As may be deduced, the repair of alkylation damage is crucial to the fidelity of DNA synthesis. The persistence of alkylation damage in tissues is well correlated with the tumorigenic ability of that compound in that tissue (Singer, 1979). The injection of MNU and ENU into young rats produced alkylation of liver and brain tissues at similar levels, however, the development of tumors took place only in the brains and not the livers of these animals (Goth and Rajewsky, 1974; Margison and Kleihues, 1975). The removal of $O^6$-alkylguanine damage in the livers of these animals was quite good, but the repair capacity of brain tissue for this same lesion was non-existent. Thus a correlation between the repair capacity of tissues and tumor initiation was also added to the various factors relating $O^6$-alkylguanine to tumor initiation. Variable rates of repair have also been found between other tissues (Nicoll, Swann, and Pegg, 1975; Pegg and Hui, 1978). The involvement of glycosylase and endonuclease activity in the repair of $O^6$-alkylguanine was discounted. On the basis of this evidence a number of sensitive assays have been elaborated in human tissues to determine the levels of DNA repair in various organs (D'Ambrosio, et al, 1984).

**Bacterial Repair of Alkylation.** In *E. coli* resistance to the mutagenic effects of alkylating agents such as MNNG has been characterized and found to be independent of the SOS repair response (Samson and Cairns, 1977; Jeggo, et al., 1977). Adaptation to the
effects of DNA alkylation in *E. coli* was linked to the production of \( \text{O}^6 \)-alkylguanine residues (Lawley and Orr, 1970), the removal of which was saturable (Schendel and Robbins, 1978). The *ada* gene for the *E. coli* enzyme has been mapped in the bacterial genome and is coinduced with the DNA glycosylase activities which repair N-alkylated purine bases (Jeggo, 1979; Sedgewick, 1982; Karran, Hjelmgren, and Lindahl, 1982; Evensen and Seeburg, 1982). However, \( \text{O}^6 \)-alkylguanine repair in bacterial DNA proved to be independent of the involvement of glycosylase or endonuclease based excision-repair pathways which had been elucidated for other types of DNA damage (Karran, Lindahl, and Griffin, 1979). In this respect, the removal of \( \text{O}^6 \)-alkylguanine residues from DNA is comparable between *ada* competent *E. coli* and mammalian cells.

**Characteristics of Alkyltransferase Enzymes.** Crude protein preparations from such bacterial cells have been used to demonstrate that the removal of the alkyl residue from the \( \text{O}^6 \) position of guanine in alkylated DNA is by direct transfer to a cysteine residue in the protein (Olsson and Lindahl, 1980) which restores guanine to its original configuration (Foote, Mitra, and Pal, 1980). Methyltransferase enzyme from all sources tested to date demonstrate "suicide kinetics"; the transfer of the alkyl group to the acceptor protein is via the formation of an irreversible covalent bond which inactivates the active site of the enzyme. The methyltransferase activity found in *E. coli* and several other bacteria (Morohoshi and Munkata, 1983; Hadden, Foote, and Mitra, 1983; Ather, Ahmmed, and
Riazuddin, 1984.) is in most respects similar in activity to the mammalian enzyme; however, the level of activity is organ and species specific (Yarosh, 1985).

The inducibility of the mammalian counterpart of the alkyltransferase enzyme is likewise variable and by no means characterized or well understood since it appears that the response of various tissue and cell types to O6-alkylation is a complex activity involving as yet unexplored mechanisms of gene regulation and activity. In fact, the mammalian systems thus far described show a range of tissue distributions corresponding to the rate of removal and tumorigenic susceptibility of the tissue (Singer, et al., 1981; Goth and Rajewsky, 1974). There is also substantive evidence that methyltransferases levels in the liver may correlate to hepatocellular differentiation and regeneration (Hesse, Mezger, and Wiebel, 1984; Pegg and Perry, 1981).

The molecular weight of purified E. coli methyltransferase has been determined at 18 kilodaltons (Demple, Jacobsson, Olsson, Robins, and Lindahl, 1982). Crossreactivity of antibodies against the 18Kd protein with the cloned 37 Kd ada protein of E. coli (Sedgewick, 1983) provided evidence that the In vivo activity exists as part of a larger peptide. The latter protein also has phosphotriester repair activity, which is lacking in the former (Margison, Cooper and Brennan, 1985). The 18 Kd substituent is obtained by cleavage of the larger molecule following cell lysis (Teo, et al., 1984), lacks phosphotriester repair activity (Margison, Ibid) and may be artifactual.
The mammalian preparations of O\textsuperscript{6}-alkyltransferase have been reported to have molecular weights ranging from 18 to 24 kD including crude human preparations (Pegg, et al., 1983, 1983; Hora, Eastman and Bresnick, 1983; Myrnes, Glercksky, Krokan, 1982; Harris, Karran, and Lindahl, 1983; Yarosh, et al., 1984). The mammalian enzyme does not have phosphotriester repair activity (Pegg, et al., 1983) and there is no conclusive evidence regarding cleavage of these enzymes. Biphasic kinetics have been reported for the repair activity of mammalian methyltransferase preparations and may be influenced by the dilution of repair activity as a property of the extent of substrate alkylation; a property which may derive from the terminal, irreversible nature of the repair activity of this enzyme, i.e., the suicide activity of this protein may progressively reduce its activity in vitro (Scicchitano and Pegg, 1982; Medcalf and Lawley, 1981; Shiloh and Becker, 1981).

The ability of bacterial methyltransferase enzyme to repair O-alkylation damage is most specific for O\textsuperscript{6}-methylguanine residues, with a concomitant methylphosphotriester repair activity (McCarthy, et al., 1983; Teo, et al., 1984). However, the size of the alkyl adduct influences the repair activity of this enzyme significantly, decreasing activity has been reported as a function of increasing chain length (Sedgewick and Lindahl, 1982; Pegg, et al., 1983; Warren and Lawley, 1980; Todd and Schendel, 1983). It has also been shown that O\textsuperscript{4}-methylthymine is repaired much more slowly than O\textsuperscript{6}-methylguanine (McCarthy et al., 1983, 1984; Ahmed and Laval, 1984). The activity of this enzyme on O\textsuperscript{4}-ethylthymine has been
demonstrated, but at much lower levels of activity than found with other O-alkylated substrates (WanI, unpublished observation) the actual kinetics of which have not been fully determined as yet. The repair of O\textsuperscript{2}-alkylthymidine is mediated via a specific glycosylase (Renard and Verly, 1980; McCarthy, Edington and Schendel, 1983). In contrast, mammalian methyltransferase appears to have minimal activity against O\textsuperscript{4}-alkylthymine and alkylphosphotriesters (Pegg, et al, 1983; Teo, et al, 1984; Yarosh, 1985).

**Mechanisms of Alklation Damage Induced Mutagenesis**

O-alkylated bases are presumed to derive their mutagenic activities from their ability to misincorporate against atypical bases during DNA synthesis, an activity which could lead to the generation of point mutations. Techniques of *in vitro* DNA strand synthesis along oligonucleotide polymers by nucleic acid polymerases have established a wealth of theoretical background pertinent to the misincorporation activities of alkylated nucleotides (Singer, 1982).

One of the first alkylated bases to be studied by *in vitro* strand synthesis along polynucleotide templates was 3-methylcytidine (Ludlum, 1970a,b). 3-Methylcytidine was incorporated into a polycytidic acid template for DNA dependent RNA polymerase. The findings of this study showed that 3-methylcytidine misincorporated adenine, uridine or cytidine into the complementary strand (Ludlum, 1970; Singer, 1982).

Subsequent studies with O\textsuperscript{6}-alkylguanine showed directed misincorporation of adenine and uridine (Gerchman and Ludlum, 1973) by RNA polymerase, and against adenine and thymine in DNA polymerase
systems (Abbott and Saffill, 1979). Numerous other miscoding
capacities of modified bases have been investigated using
polynucleotide template directed, polymerase mediated strand
synthesis.

Interestingly, O^4-alkylthymines have a great mispairing capacity
for guanine. These substituted bases are acceptable substrates for
DNA and RNA polymerases and do not unduly inhibit strand synthesis.
In contrast, O^6-alkylguanines may be inhibitory to strand synthesis
and in many cases are best incorporated by terminal deoxynucleotidyl
transferase, a template independent enzyme (Hoard and Ott, 1965).
However, synthesis arrest may be due to steric hindrance during
incorporation into repetitive purine sequences (Topal and Toorchen,
1983).

O^6-alkylguanine is fixed into an electron configuration which
allows for the formation of O^6-G-T pairs and O^6-alkylG-A pairs which
can lead to A-T transition and T-A transversion mutations (Drake,
1970; Topal and Fresco, 1976; Topal and Toorchen, 1983). Similarly,
O^4-alkylthymine lesions may also form hydrogen bonded pairs with
guanine which are stable (Singer, 1982).

Site Specific Mutagenesis

General Description. The advent of molecular biological
techniques has provided a host of site specific mutagenesis techniques
for the study of the in vivo mutagenic activity of altered bases
(Muller, et al, 1978; Shortle and Nathans, 1978; Hutchinson, et al.,
1978). These methods allow for the insertion of a substituted base or
base analog into a desired site of a DNA molecule, usually a small
plasmid, virus, or bacteriophage carrying a selective marker. The treated DNA is transfected into a cell and allowed to replicate in vivo. The replicates of the transfected DNA are isolated from selected clones and screened for the presence or absence of a mutational event. The methodologies vary in their initial approach and provide a flexibility of applications which may be selected to the relevance of the system being investigated (Green, et al., 1984).

Oligonucleotide Directed Mutagenesis. There are three basic types of approach to site specific mutagenesis. The first, oligonucleotide directed mutagenesis, utilizes an oligonucleotide containing the aberrant base being investigated (Dalbadie-McFarland et al., 1984). The oligonucleotide is hybridized to a single stranded template where it acts as a primer for completion of the synthesis of a complementary strand. The double stranded molecule is transfected into a suitable host and allowed to replicate. Replicates of the mutagenized DNA are isolated and screened for mutagenic alterations.

Primer Elongation Mutagenesis. In another strategy, an oligonucleotide primer is annealed to a single stranded DNA molecule and extended using the error prone DNA synthesis of AMV DNA polymerase to insert the base anomaly during primer elongation (Green, et al., 1984).

Gap-Misrepair Mutagenesis. Finally, gap directed misincorporation mutagenesis utilizes a gap created by limited DNA hydrolysis with the 5'-3' exonuclease activity of M. luteus DNA polymerase or the 3'-5' exonuclease activity of T4 DNA polymerase at
nicks in double stranded DNA (Shortle et al., 1978). The gap is filled in using a DNA polymerase to insert the mutagenic base misincorporation desired.

**Applications of Site Specific Mutagenesis.** The application of site directed mutagenesis was first described in characterizing the effects of N⁴-hydroxycytosine in the RNA virus Q-beta (Muller, et al., 1975). Subsequently, gap directed mutagenesis was developed to study the effects of N⁴-hydroxycytosine in plasmids containing rabbit beta-globin gene (Flavell, 1978). Gap directed misrepair mutagenesis was also used to assay the effects of sodium bisulfite deamination of cytidine in SV40 large T antigen, and in various restriction sites in the plasmid pBR322 (Shortle and Nathans, 1981; Shortle and Botstein, 1982). The mutagenicity of O⁶-methylguanine has been characterized in *E. coli* using primer extension mutagenesis in filamentous chimeric phage f1/R2028 (Topal and Toorchenh, 1984). Reversions of bacteriophage 0X174 amber mutants were generated by base misinsertions using primer elongation along RF DNA templates (Green, et al., 1984). Oligonucleotide directed mutagenesis has been used numerous times to mutagenize various genes (Dalbadle-McFarland et al., 1982; Winter et al., 1982; Carter et al., 1984).

**Experimental Hypothesis**

**Summary of background pertinent to O⁴-ethylthymine.** O⁴-Ethylthymine is a minor product of alkylation damage in DNA. However, the repair of this lesion by alkyltransferases is much lower than that for O⁶-alkylguanines. The persistence of this lesion in
the DNA of various tissues and cells may contribute to a higher level of mutagenicity compared to the relative levels of this product generated by alkylation. The mutagenicity of O\textsuperscript{4}-ethyl thymine may also relate to the ambiguity of its behavior as a template and as a substrate during DNA synthesis. The formation of base pairs with guanine during DNA synthesis, and the low specificity of methyltransferase in \textit{E. coli} for this lesion implies that the mutagenic activity of alkylation agents may be more dependent on O\textsuperscript{4}-alkylthymine. In contrast, O\textsuperscript{6}-alkylguanine, which is efficiently repaired, may play a lesser role in the overall mutagenicity of O-alkylating agents in bacteria. Furthermore, the minimal repair of O\textsuperscript{4}-alkylthymine in mammalian systems suggest that this may be the substantially mutagenic, hence carcinogenic, lesion produced by ENU, MNU, DMN and related compounds.

**Objectives.** The objective of this investigation is to use the gap misrepair mutagenesis scheme to quantify the mutagenic activity of this alkylated base alone. Because of the stringency and specificity of the restriction endonucleases, a base change due to incorporation of O\textsuperscript{4}-ethylthymidine will affect the ability of the enzyme to cleave that site. Thus, a simple, effective mutagenesis assay based on restriction endonuclease specificity may be used to characterize a mutational change. In order to accomplish this goal, O\textsuperscript{4}-ethylthymidine was incorporated into the restriction endonuclease site common to \textit{SalI} and \textit{HindIII} of the plasmid PUC8. Isolates were screened for the abrogation of restriction endonuclease cleavage to quantify mutational frequencies.
Three effects were characterized using this system; DNA polymerase 5'3'-polymerase and 3'-5' exonuclease specificities; the effect of nucleotide pool concentrations; and the effect of *E. coli* alkyltransferase treatment *in vitro* on the mutagenic efficiencies of pUC8 DNA gap misrepaired with O4-ethythymidine triphosphate.

**Thesis Statement.** Given the theoretical background already elucidated regarding the mutagenic properties of O4-alkylthymine, it may be expected that in an assay system like gap misrepair mutagenesis, O4-ethythymine lesions will cause a high rate of transition mutations at affected sites. Therefore, the hypothesis of this experimental protocol predicts that gap misrepair directed incorporation of O4-ethythymidine into the HincII and SalI sites of pUC8 will lead to the production of C-G to T-A transitions in HincII or C-G to T-A and T-A to C-G transitions in SalI. These point mutations will eliminate the ability of the cognate enzyme to cleave this plasmid at these sites, thus producing an easily characterized, restriction endonuclease resistant mutant. Finally, the influence of nucleotide triphosphate pool, DNA polymerase fidelity, and alkyltransferase activity will affect the mutation efficiency of this system. The results shown here substantiate each of these claims and provide insight into the interaction of these mechanisms with the efficiency of O4-ethythymine misincorporation mutagenesis. These results indicate that O4-ethythymine is a very powerful mutagenic lesion.
CHAPTER II
MATERIALS AND METHODS

MATERIALS

Reagents

The deoxynucleotide triphosphates; deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine triphosphate; adenosine triphosphate, thymidine, and tetrasodium pyrophosphate were obtained from Sigma. Ethylurea, sodium nitrite, silver oxide (Ag$_2$O), isopropyl bromide, carbonyldimidazole (CDI), dimethylformamide (DMF), para-nitrophenylphosphate (PNPP), and sodium ethoxide were procured through Aldrich. Gel electrophoresis was performed in electrophoretic grade agarose from SeaKem. Ethidium bromide from Boehringer-Mannheim Biochemicals was used for gel staining and in plasmid nicking reactions. All other reagents were standard laboratory items.

Bacteriological Supplies

General Supplies. The host bacterium E. coli K-12 strain JM83 (ara, lac-pro, thi, strA, 80, lacZM15) and the plasmid pUC8 (Figure 4) were obtained through Bethesda Research Laboratories. Difco Laboratories agar, bacto-peptone, bacto-tryptone, and yeast extract were used in the bacteriological media described here. A lactose operon inducer, isopropyl-beta-D-thiogalactoside (IPTG), and a color indicator substrate, 5-bromo-4-chloro-3-indoly1-beta-D-galactoside
Figure 4. The plasmid pUC8 containing the genes for beta-lactamase (ampicillin resistance) and beta-galactosidase (lac Z'). The lac Z' gene contains the polylinker region in which are the Sall and HindIII sites.
(Xgal) were purchased from Bethesda Research Laboratories. Sodium ampicillin was purchased from Sigma. Lysozyme and proteinase-K were purchased from Worthington Enzymes and Bio-Rad, respectively.

**Media.** All media used were prepared according to standard, published techniques (Messing, 1983). YT broth consisted of bacto-tryptone (8 g/L), yeast extract (5 g/L), and NaCl (5 g/L), pH 7.4. Hard and soft YT agar were prepared by adding 15 g/L or 6 g/L agar, respectively, to YT broth and dissolving at 100°C. LB broth was prepared from bacto-peptone (10 g/L), yeast extract (5 g/L), and NaCl (10 g/L), pH 7.5. All media were sterilized by autoclaving for 15 minutes and then stored at room temperature. Agar media was reheated to melting and poured in 10 ml volume into bacteriological petri dishes. Standard aseptic techniques were observed.

**Nucleic Acid Enzymes**

AMV Reverse Transcriptase (DNA polymerase), *E. coli* DNA polymerase Large ("Klenow") Fragment, and the restriction endonucleases; *SalI*, *HincII*, *TagI*, *EcoRI*, *BamHII*, *AccI*, came from Bethesda Research Laboratories. *T*4 DNA ligase, *T*4 DNA polymerase, and the restriction endonucleases; *PstI*, *MboI*, *ClaI*, *HpaI*, were produced by New England Biolabs. *Micrococcus luteus* DNA polymerase and RNAse A were available from Sigma. Crude wheat shoot phosphotransferase was prepared from germinated locally available wheat seed.

**Chromatographic Materials**

Preparative and analytical silica gel and cellulose thin layer chromatography (TLC) plates were purchased from Kodak. The column chromatography materials were obtained from the following sources:
DEAE-Sephadex A25, was obtained from Pharmacia; Dowex 50 WX4 from Bio-Rad; silicic acid from Sigma, and nitrocellulose from Whatman. Affinity chromatography was executed on acridine yellow bisacrylamide gel from Boehringer-Mannheim Biochemicals.

SYNTHESIS OF O4'-ETHYLTHYMIDINE TRIPHOSPHATE

General Protocol

O4'-ethylthymidine triphosphate was prepared from thymidine in three major steps. The first stage of synthesis involved preparation of O4'-ethylthymidine by alkylation of the O4'-position of thymidine. O4'-alkylation was accomplished by two methods: the first was by reacting thymidine with diazoethane followed by separation of the O4'-ethyl product; the second procedure involved the production of an O4'-isopropyl derivative which was in turn reacted with sodium ethoxide to produce the desired substituent. Comparative yields between the two ethylation methods were analyzed. The O4'-ethyl product was enzymatically converted to a monophosphate by wheat shoot phosphotransferase using PNPP as a phosphate donor. Purified O4'-ethylthymidine monophosphate was reacted with sodium pyrophosphate to yield the final product, O4'-ethylthymidine triphosphate (O4'-EtTTP) (Mehta, and Ludlum, 1978; Singer, Fraenkel-Conrat, and Kusmierek, 1978; Singer, Sagl, and Kusmierek, 1983).

Preparation of O4'-Ethylthymidine with N-Ethyl, N-Nitrosourea

Synthesis of N-Ethyl, N-Nitrosourea. A cold aqueous solution containing 55.40 g ethylurea and 43.44 g sodium nitrite was prepared in 160 ml distilled water to which was added 40 ml 0.31 M H2SO4
(Arndt, 1948; Kusmierek and Singer, 1976; Farmer et al., 1976). The reaction was kept chilled in an ice bath and allowed to proceed for 30 minutes during which time solid ENU precipitate formed. The ENU precipitate was collected by filtering the reaction mixture through a Buchner funnel, followed by washing with ice cold water. The crude ENU was dissolved in 300 ml anhydrous methanol at 40°C. The ENU was crystallized from the methanol solution by storing at -20°C overnight. The crystals were filtered and collected on a Buchner funnel, washed with cold anhydrous methanol, and dessicated under vacuum.

Synthesis of diazoethane. In a 150 ml round bottomed flask immersed in an ice bath, 10 g of ENU and 100 ml diethyl ether were added to 25 ml of aqueous 50% (w/v) (Singer and Kusmierek, 1976). The gaseous diazoethane was trapped in the ether layer giving it a yellow color. After completion of the reaction, the ether layer containing diazoethane was distilled at 55°C into a round bottom flask and stored at 4°C until used the same day.

Alkylation of Thymidine with Diazoethane. The alkylation of thymidine by diazoethane was performed according to published procedures (Farmer et al., 1973; Singer and Kusmierek, 1976). To a chilled (0°C) suspension of 1 g thymidine in 500 ml anhydrous methanol was added 200 ml (10 mMol) etheral diazoethane which had been prepared as described above. The reaction mixture was incubated at 25°C with vigorous stirring for 4 hours. The mixture was evaporated to 50 ml in a rotary flash evaporator and applied to a 2 x 30 cm sillicic acid column in acetone to resolve the O4-ethyl
substituent from the other reaction products, primarily N-3 and $O^2$-ethyl substituents. The $O^4$-ethylthymidine fraction in acetone was further purified on preparative TLC plates developed in acetone/benzene (60:40, v/v). The band corresponding to the $R_f$ value of $O^4$-ethylthymidine (0.33) was scraped off and recovered by washing three times with acetone. The excess solvent was evaporated under vacuum in a rotary flash evaporator. The TLC purification was repeated three times and pure $O^4$-ethylthymidine was crystallized from acetone/benzene. The purity of the product was determined from UV absorption spectra (max =281 nm, min =240 nm) and melting point (174°C).

Preparation of $O^4$-Ethylthymidine via $O^4$-Isopropyl Derivatization

$O^4$-Isopropylation Reaction. The preparation of $O^4$-ethylthymidine via an $O^4$-isopropyl derivative was performed following a modification of a protocol for the preparation of $O^4$-methylthymidine (Singer, Sagl, and Kusmierek, 1983). In this preparation, 2.4 g of thymidine were reacted with 5 ml of isopropyl bromide and 2.5 g of Ag$_2$O in 50 ml of isopropanol for 48 hours at 37°C. After 48 hours 2.5 ml of isopropyl bromide and 1.25 g Ag$_2$O were added and the reaction was incubated for 24 more hours. Purification of the $O^4$-isopropyl derivative was achieved on preparative silica gel plates developed in chloroform/isopropanol (80:20). The $O^4$-isopropylthymidine band ($R_f$=0.47) was scraped off and extracted 3 times with isopropanol. The isopropanol solution was concentrated to 10 ml and crystallized from benzene.
Ethylation of the O4-Isopropyl Derivatized Site. To ethylate the O4-position of the isopropyl derivative, 500 mg of O4-isopropylthymidine crystals were dissolved in 5 ml anhydrous ethanol, to which was added 1 ml sodium ethoxide. The reaction mixture was incubated at 37°C for 48 hr, stirring constantly. The reaction mixture was evaporated under vacuum to a reduced volume of 1 ml. The 1 ml volume was separated on a preparative silica gel thin layer chromatography plate developed in chloroform/isopropanol (80:20). The band corresponding to O4-ethylthymidine was scraped and washed with acetone to recover the product. The TLC purification was repeated 3 times. The purity of this compound was determined from melting point and UV absorbance spectra as described above.

Preparation of O4-Ethylthymidine Monophosphate

O4-ethylthymidine monophosphate was prepared using enzymatic phosphorylation with wheat shoot phosphotransferase enzyme, using PNPP as a phosphate donor (Gizlewicz and Shugar, 1975).

Preparation of Wheat Shoot Phosphotransferase. The wheat shoot phosphotransferase enzyme was prepared from wheat kernels which had been germinated in the dark on cellulose filter paper saturated with distilled water at room temperature for 5 to 7 days. The cotyledons were removed and discarded; the shoots were homogenized with a Waring blender in a 10 fold volume of 0.02 M sodium acetate buffer (pH 4.5). The homogenate was centrifuged at 4000 x g, and the cleared supernatant was stored at -20°C.
Enzymatic Phosphorylation of $^{04}$-Ethylthymidine.

$^{04}$-ethylyimidine and 1.2 g para-nitrophenylphosphate were dissolved in a reaction mixture containing 6 ml wheat shoot phosphotransferase preparation, 3 ml 1 M sodium acetate (pH 4.5). The reaction mixture was incubated for 16 hours at 27°C. At the end of that time, the reaction mixture was titrated to pH 8 by the dropwise addition of 1M ammonium hydroxide. The mixture was filtered through a cellulose filter and loaded onto a DEAE-Sephadex column (1.5 x 40 cm) equilibrated with 50 mM triethylammonium bicarbonate (TEAB) buffer (pH 8.0).

Preparation of TEAB buffer. TEAB buffer was prepared by dissolving triethylamine in water by dropwise addition, constantly stirring and bubbling carbon dioxide through the solution (Glezlewicz and Shugar, 1975). This was continued until the TEA was completely dissolved in a 0.5 M stock solution (pH 8).

Column Chromatography of $^{04}$-Ethylthymidine Monophosphate.

Purification of $^{04}$-ethylyimidine monophosphate was achieved by elution from the DEAE-sephadex column with a 0 to 0.5 M HCO$_3^-$ gradient. Fractions were monitored at 280 nm and those corresponding to a peak at this wavelength were pooled and evaporated under vacuum. $^{04}$-ethylyimidine monophosphate eluted separate from TMP and unreacted constituents.

Reaction of $^{04}$-Ethylthymidine Monophosphate and Pyrophosphate

Preparation of Tributylammonium Pyrophosphate. $^{04}$-Ethylthymidine monophosphate was converted to a triphosphate by reacting it with tributylammonium pyrophosphate. Tributylammonium pyrophosphate was
prepared by dissolving 446 g (1 mmol) tetrasodium pyrophosphate in 5 ml water. This solution was passed through a Dowex WX4 column in pyridinium form (Dowex WX4 was washed with 6N HCl and placed in 20% pyridine overnight). 240 ul of tributylamine was added to the eluate to convert the pyrophosphate to the tributylammonium salt. This salt was dried by two evaporations from anhydrous pyridine and dissolved in 10 ml dimethylformamide (Hoard and Ott, 1964; Singer, Sagl, and Kusmlerek, 1983).

Preparation of O^4^-Ethylthymidine Monophosphate Phosphorimidazolidate Salt. Crystalline O^4^-ethylthymidine monophosphate was dissolved in 5 ml 20% (v/v) pyridine and passed through a pyridinium Dowex WX4 column (1x10 cm). The eluate was converted to the tributylammonium salt by adding 10 ul of TEA. The TEA salt of O^4^-ethylthymidine monophosphate was dried with pyridine and dissolved in 250 uL DMF to which was added 32mg of carbonyldilimidazole (CDI) in 400 uL DMF. After incubation for 4 hr at 25°C under nitrogen, 50 uL anhydrous methanol was added to destroy unreacted CDI.

Preparation and Purification of O^4^-Ethylthymidine Triphosphate. O^4^-ethylthymidine monophosphate, in imidazolate form at this point, was reacted with 2 ml tributylammonium pyrophosphate and incubated in a nitrogen atmosphere for 22 hr at 25°C. A white precipitate, the O^4^-Ethylthymidine triphosphorimidazollum salt, was dessicated and dissolved in 50 ml 50 mM TEAB buffer, pH 8. The sample was loaded onto a DEAE-Sephadex column (1.5 x 40 cm) equilibrated in 50 mM TEAB buffer, pH 8. The triphosphate was purified using a 0-1.0 M gradient.
of triethylammonium bicarbonate (pH 8.0). The fractions were monitored for absorbance at 280 nm. The peak which eluted at this wavelength in 0.6 M TEAB buffer, corresponded to O\textsuperscript{4}-ethylthymidine triphosphate product.

The TEAB was removed by repeated evaporation from 70% ethanol. The purity of the O\textsuperscript{4}-EtTTP was determined using UV absorbance (280 nm max, 241 nm min). The final O\textsuperscript{4}-EtTTP was stored at -20\degree C, in 50% ethanol (v/v) as a 1 mM stock solution (Hoard and Ott, 1964).

MUTAGENESIS OF pUC8 BY GAP-MISREPAIR MUTAGENESIS MISINCORPORATION OF O\textsuperscript{4}-ETHYLTHYMIDINE.

General Gap-Misrepair Mutagenesis Protocol

Gap-misrepair mutagenesis was performed following a modification of established procedures (Shortle and Nathans, 1981). The restriction endonuclease site mutagenized here was a hexanucleotide segment of the polylinker region insert in the lacZ gene of pUC8 recognized by Sal\textit{I}, Hin\textit{c}l\textit{I}, and Acc\textit{I}. This sequence, 5'-GTCGAC-3', is cleaved by these enzymes as follows: Sal\textit{I}, 5'G//TCGAC-3'; Hin\textit{c}l\textit{I}, 5'-GTPy//PuAC-3'; Acc\textit{I}, 5'-GT//CGAC-3' (Messing, 1983).

For use in gap misrepair mutagenesis, this sequence was nicked using Sal\textit{I} or Hin\textit{c}l\textit{I} by including an appropriate amount of ethidium bromide in the enzyme buffer to inhibit double strand scission of the molecule by the endonuclease (Parker, Watson and Vinograd, 1977; Shortle and Nathans, 1981) (Plate 1., Plate 2.). In the absence of deoxynucleotide triphosphates, the nick was converted into a 6-10 base pair gap by the 5'-3' exonuclease activity of \textit{M. luteus} DNA polymerase (Shortle, et al., 1982). The gapped plasmid was purified
on an acridine yellow column and used for the O4-ethylthymidine incorporation reactions detailed below (Shortle and Nathans, 1981).

**DNA Polymerase 5'-3' Synthesis Specificities.** The initial mutagenesis study investigated the effect of DNA polymerase 3'-5' exonuclease (proofreading) activity during 5'-3' polymerase DNA synthesis on the efficiency of mutational frequency in the treated plasmid. Of the DNA polymerases used, AMV reverse transcriptase has no proofreading activity (Zakour, James, and Loeb, 1984), *M. luteus* DNA polymerase has weak proofreading ability (Shortle and Botstein, 1983), and *E. coli* DNA polymerase Large Fragment has strong proofreading ability (Shortle, Brutlag, and Kornberg, 1972). Thus the mutagenic frequency of gap repaired plasmid should be a function of the ability of the DNA polymerase used to correct misinsertions during 5'-3' DNA synthesis. That is to say, that the ability of a DNA polymerase to proofread during synthesis is central to its ability to faithfully reproduce the genetic code of an organism. The Incorporation reactions included 100 uM concentrations of dATP, dCTP, dGTP, TTP, and O4-EtTTP. Misinsertion of O4-EtTTP was expected to vary as a function of proofreading activity, AMV DNA polymerase was expected to misinsert O4-ethylthymine with a much higher frequency than the bacterial polymerases used in this study. The gap repaired plasmid was covalently closed by T4 DNA liggase (Figure 5., Figure 6.). The transformation of the *E. coli* was done directly from these incorporation mixtures. The number of transformants were scored and the relative transformation efficiencies were calculated on the basis of total transformants per ug of DNA.
Plate I. Titration of ethidium bromide concentrations in the nicking reaction of SalI. Each well contains 200 ng pUC8, 1 Unit SalI, in 25 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, 100 mM NaCl, 100 ug/ml BSA, 1 mM DTT. Lanes A and H contain a control samples showing Form I, II, and III DNA. Lanes B-G contain 10, 20, 30, 40, 50, 60 ug/ml ethidium bromide.
Plate I

FORM I

SalI
Plate II. Titration of ethidium bromide concentrations in the nicking reaction of Hincl. Each well contains 200 ng pUC8, 1 Unit SalI, in 25 mM Tris-HCl (pH 7.8), 2 mM MgCl2, 50 mM NaCl, 100 ug/ml BSA, 1 mM DTT. Lane A contains a control sample showing Form III DNA. Lanes B-G contain 20, 30, 40, 50, 60, 10 ug/ml ethidium bromide.
Figure 5. The basic gap misrepair mutagenesis scheme of HincII.
GAP MISREPAIR OF SalI

- 5' GTCGAC 3' -
- 3' CAGCTG 5' -

NICKING REACTION

\[ \text{SalI} + \text{EtBr} \]

- 5' GTCGAC 3' -
- 3' CAGCTG 5' -

GAPPING REACTION

\[ \text{M. Luteus DNA Polymerase} + \text{Mg}^{2+} \]

- 5' G...... PO_4^{3-} 3' -
- 3' CAGCTG 5' -

MISINCORPORATION REACTION

\[ \text{DNA Polymerase} + \text{dTTP, dNTPs: Ligase + ATP} \]

- 5' GTTGAT 3' -
- 3' CAGCTG 5' -

transform E. coli

Figure 6. The basic gap misrepair mutagenesis scheme of SalI
The Effect of Nucleotide Pool Concentration. Because $O^4$-EtTTP can incorporate across from adenine or guanine during DNA polymerase mediated strand synthesis, the effect of *in vitro* dNTP pool composition on the mutagenic frequency of $O^4$-EtTTP gap misrepaired pUC8 was investigated. The same protocols for the first section were followed, only the molar amounts of dCTP, TTP, and $O^4$-EtTTP were varied with respect to each other.

The Effect of $T_4$ DNA Polymerase 3'-5' Exonuclease Activity. Apart from 5'-3' polymerase activity, the 3'-5' exonuclease activity of proofreading DNA polymerases may also be a source of misinsertion mutagenesis. $T_4$ DNA polymerase has one of the strongest 3'-5' exonuclease activities known, and this property is often used in 32P-dNTP labeling reactions of DNA molecules by a 3'-5' exchange reaction. In this study, the *HincII* nicked plasmid was incubated with $T_4$ DNA polymerase in the presence of $O^4$-EtTTP to determine the mutagenic efficiency of 3'-5' exonuclease misincorporation at the two pyrimidine loci immediately 3' to the nick. This protocol was not performed on *SalI* nicked DNA since no pyrimidine residues within this site lie 3' to the nick.

The Effect of Alkyltransferase Treatment. Finally, the effect of *E. coli* methyltransferase on the mutagenic efficiency of $O^4$-ethylthymine misincorporation was assayed by the *in vitro* incubation of $O^4$-ethylthymine containing plasmid with a partially purified preparation of *E. coli* methyltransferase prepared from *E. coli* B strain BS21 (Yale *E. coli* Genetic Stock Repository) by the procedure of Ahmmed and Laval (Ahmmed and Laval, 1978).
Treated plasmid from the above protocols was transfected into the host bacterium by the CaCl$_2$ coprecipitation method. Transformants were selected for ampicillin resistance, and select clones were cultivated. Plasmid isolates from these transformants were screened for resistance to the relevant restriction endonuclease. Resistant clones were then tested with additional restriction endonucleases in order to discern the nature of the base change in the restriction endonuclease site. Detailed procedures are listed as follows.

**Transformation of E. coli**

**Preparation of Strain JM 83 Stock Cultures.** Escherichia coli K-12 strain JM 83 was obtained from stocks frozen at -20°C by streaking onto YT hard agar plates, followed by incubation at 37°C for 24 to 48 hours. A single colony was picked off and inoculated into 50 ml LB broth and grown to mid log phase ($A_{590nm} = 0.4-0.6$), 10 ml aliquots of this culture were stored at -20°C in 15% glycerol. Transformation of E. coli with pUC8 was done following established procedures (Cohen, Chang, and Hsu, 1973; Hanahan, 1983).

**Preparation of Competent Cells.** An inoculum from stock JM 83 cultures was grown to an $A_{590}= 0.6$ in 200 ml LB broth. This culture was centrifuged at 4,000 x g for 15 minutes at 4°C. The supernatant was decanted and the pellet was resuspended in 10 ml chilled competency buffer; 50 mM CaCl$_2$, 10 mM Tris-HCl, pH 8.0. The bacterial suspension was centrifuged at 4,000xg for 7 minutes at 4°C. The supernatant was decanted and the bacterial pellet was
suspended in 2 ml competency buffer and stored overnight at 0°C. For transformation, 200 ul aliquots of competent bacterial suspension were placed in sterile 3 ml vials with 200 ng supercoiled pUC8.

**Incorporation of Plasmid.** Vials containing competent E. coli JM 83 in competency buffer were kept on ice for 30 minutes, shaken occasionally, and then heat pulsed at 42°C for 2 minutes by suspending the vials in a warm water bath. The bacteria were left to stand 10 minutes at 25°C to allow completion of plasmid uptake. A 1 ml volume of LB broth was added to the bacteria and they were incubated for 1 to 2 hours to allow for the expression of the plasmid beta-lactamase gene. A 200 uL volume of the treated E. coli culture was added to 3 ml liquid (42°C) soft YT agar containing 25 ug/ml ampicillin, 200 mM IPTG, and 500 mM Xgal. The bacteria were mixed by gentle swirling. The soft agar was plated on top of 10 ml solid hard YT agar containing an equal composition of ampicillin, IPTG, and Xgal as the soft agar. The soft agar was allowed to solidify and incubated at 35°C for 24-48 hours.

Single dark blue colonies were picked off and inoculated into individual culture tubes containing 4 ml LB broth with 50 ug/ml ampicillin and incubated overnight at 37°C. These colonies were selected for their ampicillin resistance, and their ability to successfully produce beta-galactosidase. The importance of the second selection factor is necessary since the polylinker region of pUC8 is inserted in the Lac operon of the plasmid. Hence, selection of blue colonies circumvented the possibility of using a defective plasmid in
the mutagenesis studies (Messing, 1983). To the 4 ml stock cultures was added 1 ml sterile 50% (v/v) glycerol, followed by gentle swirling and storage at -20°C.

**Plasmid Isolation.** Fresh plasmid containing cultures were obtained by streaking out frozen transformed stock onto solid hard YT agar plates containing 50 ug/ml ampicillin, 200 mM IPTG, and 500 mM Xgal, Incubating for 24 to 48 hours at 37°C. Single blue colonies were selected and grown up in 200 ml LB broth containing 50 ug/ml ampicillin. These cultures were used for preparation of wild type pUC8 (Holmes and Quigley, 1981). These cultures were grown to mid log phase, centrifuged at 4,000 x g. The media was decanted and the bacteria were suspended in 1/5 volume of STET buffer [20% sucrose (w/v), 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 5% Triton-X 100 (v/v)]. Lysozyme was added to a concentration of 1 mg/ml and the mixture was incubated for 10 minutes at 25°C. The preparation was immersed in boiling water for 90 seconds, removed, chilled on ice and centrifuged at 12,000 x g for 15 minutes. The pellet of bacterial debris was discarded and the supernatant was saved, one volume of chilled (-20°C) isopropanol was added, and the mixture was stored for 12 hr at -20°C.

The isopropanol precipitate was pelleted by centrifugation at 12000 x g for 20 minutes. The supernatant was discarded and the pellet was saved and desiccated. The crude plasmid pellet was resuspended in 10 ml TE buffer (100 mM Tris-HCl, 50 mM EDTA, pH 8.0). RNAse A (10 mg/ml in TNE) was added to a final concentration of 50 ug/ml. The mixture was incubated 15 minutes at 37°C. Subsequently,
SDS was added to 0.1% (w/v) final concentration and the mixture was incubated at 65°C for 15 minutes with 50 ug/ml of Proteinase K. The DNA was extracted with an equal volume of TE equilibrated phenol/chloroform/isoamyl alcohol (50:48:2), vortexed, and microfuged at 140000 x g for 5 minutes. The aqueous layer was removed and the organic layer was reextracted with an equal volume of TE buffer, vortexed and centrifuged. The aqueous layer from this extraction was pooled with the first and extracted two more times with an equal volume of chloroform/isoamyl alcohol (24:1). The aqueous fraction was brought to 300 mM sodium acetate by the addition of 1/10 volume of 3M sodium acetate (pH 4.5). Two volumes of chilled ethanol (-20°C) were added and the mixture was kept at -20°C for 12 hr. The DNA precipitate was pelleted by centrifugation at 5000 x g, dessiclated and resuspended in a minimal volume of TE buffer. Treatment of the crude pellet with RNase A followed by proteinase K digestion, phenol extraction, ethanol precipitation, and centrifugation was repeated once.

Preparation of Supercoiled Plasmid DNA. Supercoiled (Form I) pUC8 was purified from nicked and linear species by treating a 200 ug suspension of plasmid in TE buffer with 100 mM NaOH, followed by incubation for 15 minutes at 37°C, and titration to pH 8 by the dropwise addition of 2M Tris-HCl (pH 5). This mixture was chromatographed through a 1x2 cm nitrocellulose column equilibrated in TEN buffer (100 mM Tris-HCl, 50 mM EDTA, 20mM NaCl) (Wanl and Hart, 1981). The eluate contained only supercoiled DNA as determined by electrophoresis of an aliquot of this preparation through a 1.3%
agarose gel in Tris acetate buffer (125 mM Tris-acetate, pH 7.5, 50 mM EDTA, 200 mM NaCl). The plasmid containing eluate was ethanol precipitated, pelleted, and resuspended in TE buffer to a final concentration of 1 ug/ml. The final concentration was determined by electrophoresis of aliquots against known pUC8 standards.

**Agarose Gel Electrophoresis**

1.5% agarose gels were prepared by melting agarose (Seakem) in Tris-Acetate buffer at 100°C. Slab gel electrophoresis was performed on a 20 cm x 20 cm horizontal apparatus. All samples were loaded into 25 ul gel wells in a buffer containing 5% glycerol, 0.2% bromophenol blue, 0.1% SDS, 10 mM Tris-HCl (pH 7.5), 5 mM EDTA. Electrophoresis was performed at 75-100 volts, 35 mA until the indicator dye had run the length of the gel. The gels were stained in 500 ng/ml ethidium bromide for 45 minutes, then destained in distilled water for 25 minutes. DNA bands were visualized on a UV transilluminator and photographed at 15 second exposures with polaroid 667 film, using a Polaroid camera.

**SalI Nicking Reactions**

10 ug of supercoiled pUC8 was dissolved in buffer containing 25 mM Tris-HCl (pH 7.8), 10 mM MgCl2, 100 mM NaCl, 100 ug/ml BSA, 1 mM DTT, 25 ug/ml ethidium bromide. Following a 4 hour incubation at 37°C, the reaction was stopped by adding EDTA to a final concentration of 100 mM. The nicked plasmid was isolated by phenol extraction and ethanol precipitation (Kovacs, Gregory, and Butterworth, 1984).
**HincII Nicking Reaction**

10 ug of supercoiled pUC8 was dissolved in buffer containing 25 mM Tris-HCl (pH 7.8), 2 mM MgCl₂, 50 mM NaCl, 100 ug/ml BSA, 1 mM DTT, 10 ug/ml ethidium bromide. Following a 4 hour incubation at 37°C, the reaction was stopped by adding EDTA to a final concentration of 100 mM. The nicked plasmid was isolated by phenol extraction and ethanol precipitation (Kovacs, Gregory, and Butterworth, 1984).

**Gapping Reaction**

In a 25 ul volume of 70 mM Tris-HCl (pH 8.0), 7 mM MgCl₂, 1 mM 2-mercaptoethanol, 2 ug of nicked pUC8 (either Hinc II or Sal I) was incubated at room temperature for 60 minutes in the presence of *Micrococcus luteus* DNA polymerase to create a 5'-3' gap of approximately 6-10 nucleotides. The reaction was stopped by adding EDTA to a concentration of 100 mM. The gapped DNA was phenol extracted and ethanol precipitated.

**Purification of Gapped Plasmid.** Purified gapped plasmid was obtained by dissolving the precipitated DNA from the gapping reaction in 200 ul of 10 mM sodium citrate (pH 6.0), 500 mM EDTA, 200 mM NaCl, 2 ug/ml ethidium bromide. This suspension was loaded on a 200 ul acridine yellow column equilibrated with the same buffer. The column was washed with 10 mM sodium citrate (pH 6.0), 500 mM EDTA, 200 mM NaCl, and the plasmid was eluted with the same buffer containing 500 mM NaCl. The plasmid was ethanol precipitated in the presence of 100 ng carrier t-RNA.
Misincorporation Reaction: The Effect of Polymerase Specificity

The misincorporation reactions were performed using strong, weak- or non-proofreading DNA polymerases; E. coli DNA polymerase "Klenow" fragment, M. luteus DNA polymerase or AMV DNA Polymerase (reverse transcriptase), respectively. In a 25 ul reaction volume containing 1 unit of the desired enzyme, 200 ng of gapped pUC8, 150 units of T₄ DNA ligase were added to a buffer containing, 60 mM Tris-HCl (pH 8.0), 20 mM 2-mercaptoethanol, 1 mM MgAc₂, 2 mM MnCl₂, gelatin (100 ug/ml), 0.5 mM ATP, 100 uM dGTP, dATP, dCTP and O⁴-EtTTP. Two other preparations were made: one with all of the normal dNTPs but in the absence of O⁴-EtTTP; the other with 100 uM EtTTP, but no dCTP or TTP. These reactions were incubated at 25°C for 16 hours and terminated by the addition of 5 ul 50 mM EDTA. The plasmid from this reaction was used to transform E. coli JM83.

Nucleotide Pool Concentration Effects

The same DNA polymerases as before were used, only the respective dNTP concentrations in the polymerase buffer containing 1 unit of the desired enzyme, 200 ng of gapped pUC8, 150 units of T₄ DNA ligase, 60 mM Tris-HCl (pH 8.0), 20 mM 2-mercaptoethanol, 1 mM MgAc₂, 2 mM MnCl₂, gelatin (100 ug/ml), 0.5 mM ATP, was varied.

The Effect of Equimolar Concentrations. The dNTPs; dATP, dCTP, dGTP, TTP, and O⁴-EtTTP were all included at 100 uM concentrations.

The Effect of O⁴-EtTTP Concentrations. 100 uM each of dATP, dCTP, dGTP, and TTP was included. The concentration of O⁴-EtTTP was 0, 1, and 10 uM in the respective reactions.
The Effect of TTP Concentrations. 100 μM each of dATP, dCTP, dGTP, and O4-EtTTP was included. The concentration TTP of was 0, 1, and 10 μM in the respective reactions.

The Effect of dCTP Concentrations. 100 μM each of dATP, dGTP, TTP, and O4-EtTTP was included. The concentration of dCTP was 0, 1, and 10 μM in the respective reactions.

The Efficiency of O4-EtTTP Misincorporation Mutagenesis in the Absence of dCTP and TTP. 100 μM each of dATP, dGTP, and O4-EtTTP was included. No TTP or dCTP was added.

These reactions were incubated at 25°C for 16 hours. The DNA in the reaction mixtures was used to transform *E. coli* JM 83.

*E. coli* Alkyltransferase Treatment

200 ng of pUC8 gap misrepaird with 100 μM each of dATP, dGTP, and O4-EtTTP, in the absence of dCTP and TTP by AMV DNA polymerase was used in this study. Both *Hin* and *Sal* gapped plasmids were studied in separate experiments. The *E. coli* Alkyltransferase preparation, 12 mg protein/ml was added in 40 ul reaction volumes containing plasmid preparation in a buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, 1 mM 2-mercaptoethanol, and 5% glycerol. These mixtures were incubated 20 minutes at 37°C. The DNA from this preparation was directly used to transform *E. coli* JM 83.

T4 DNA Polymerase 3'-5' Exonuclease Misinsertion Mutagenesis

200 ng *Hin* nicked plasmid was incubated with 0.5 Unit T4 DNA polymerase in the presence of 100 μM O4-EtTTP, in a buffer containing, 60 mM Tris-HCl (pH 8.0), 20 mM 2-mercaptoethanol, 1 mM
MgAc₂, 2 mM MnCl₂, gelatin (100 ug/ml), 0.5 mM ATP, for 10 minutes at 37°C. T₄ DNA ligase was added and the mixture was incubated 4 hours at 4°C.

The Effect of Competing Pyrimidines on the Mutagenic Efficiency of T₄ DNA Polymerase 3'-5' Exonuclease. The same reaction conditions were maintained only 10 and 100 uM concentrations of TTP and dCTP were added individually in separate reactions.

Alkylation Effects. Alkylation was applied as described above to a 200 ng preparation from each of the T₄ DNA polymerase studies. The conditions were the same as those given above.

Analysis of Mutants

Transformed E. coli JM 83 were selected by ampicillin resistance and restreaked twice to assure the segregation of mutant and wild type phenotypes. Ampicillin resistant colonies were selected and grown in 25 ml LB broth with 25 ug/ml ampicillin. Plasmid was harvested from these cultures and screened for cognate restriction endonuclease resistance. In a total volume of 25 ul, 200 ug of plasmid isolate was suspended in a buffer containing 25 mM Tris-HCl (pH 7.8), 100 mM NaCl, 10 mM MgCl₂, 100 mg/ml BSA, 1 mM DTT, to which 1 Unit of the desired restriction endonuclease was added (Sal I, Pst I, Taq I, or BamH I). Hinc II sensitivity was assayed in 25 mM Tris-HCl (pH 8.0), 50 mM NaCl, 2 mM MgCl₂, 100 ug/ml BSA, 1 mM DTT. Restriction endonuclease resistance was determined by the failure of the respective restriction endonuclease to convert form I DNA to form III DNA, the wild type plasmid was converted to form III, linear DNA.
CHAPTER III
RESULTS

THE INTERACTION OF POLYMERASE 3'-5' EXONUCLEASE PROOFREADING ACTIVITY ON THE MUTAGENICITY OF O4-ETHYLTHYMINE MISINCORPORATION

Mutation Frequencies

Equimolar dNTP concentrations; HincII Gap Misrepair. Equimolar concentrations of dCTP, TTP, and O4-EtTTP in the incorporation mixture yielded restriction endonuclease resistant mutants which reflected the 3'-5' exonuclease proofreading ability of the DNA polymerase used in the In vitro repair synthesis reaction (Plate III). Total numbers of transformants and screened colonies are listed in Table 6.

Inclusion of O4-EtTTP along with the normal pyrimidine deoxynucleotide substrates at 100 mM concentrations most efficiently mutagenized the Hinc II site of pUC8 when gap misrepaired with AMV DNA polymerase. The mutation frequency obtained under these conditions was 4%. M. luteus DNA polymerase, and E. coli DNA polymerase Large Fragment yielded respectively, 3% and 2% mutation frequencies (Table 1). Analysis of the adjacent restriction endonuclease sites: BamHI, 5'-GGATCC-3' (3' to HincII), and PstI 5'-CTGCAG-3' (5' to HincII), for resistance to cleavage by the cognate enzyme was performed on the HincII resistant isolates. No BamHI resistant samples were found.
Plate III. Randomly selected HincII resistant mutants. Lanes A, C, E, and G contain 200 ng pUC8 controls in TE buffer. Lanes B, D, F, and H contain 200 ng pUC8 treated with 1 Unit HincII in the appropriate Endo.R. buffer. Representative wild type plasmid which is sensitive to Endo.R. HincII (Lane B) and Endo.R. resistant mutant isolates (Lanes D, F, and G) are shown.
Plate III
Table 1
The Relative Efficiencies of Different DNA Polymerases in O^4-Ethylthymidine Misincorporation Mutagenesis

<table>
<thead>
<tr>
<th></th>
<th>a. DNA polymerase reaction contained dATP, dGTP, dCTP, and TTP at 100 uM each. No O^4-EtTTP was added.</th>
<th>b. DNA polymerase reaction contained dATP, dGTP, dCTP, TTP, and O^4-EtTTP, 100 uM each.</th>
<th>c. DNA polymerase reaction contained dATP, dGTP, and O^4-EtTTP, 100 uM each. No dCTP or TTP was added.</th>
</tr>
</thead>
<tbody>
<tr>
<td>H\text{inc}ll site</td>
<td>E. coli polymerase 0</td>
<td>1</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>M. luteus polymerase 0</td>
<td>3</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>AMV polymerase 0</td>
<td>5</td>
<td>47</td>
</tr>
<tr>
<td>Sall site</td>
<td>E. coli polymerase 0</td>
<td>3</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>M. luteus polymerase 0</td>
<td>5</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>AMV polymerase 0</td>
<td>15</td>
<td>48</td>
</tr>
</tbody>
</table>

a. DNA polymerase reaction contained dATP, dGTP, dCTP, and TTP at 100 uM each. No O^4-EtTTP was added.
b. DNA polymerase reaction contained dATP, dGTP, dCTP, TTP, and O^4-EtTTP, 100 uM each.
c. DNA polymerase reaction contained dATP, dGTP, and O^4-EtTTP, 100 uM each. No dCTP or TTP was added.
d. 100 transformants were screened in each sample shown.
The only PstI resistant sample was found in the AMV polymerase treated group. *M. luteus* DNA polymerase and *E. coli* polymerase treated samples yielded no PstI resistant isolates (Plate 5).

**Control Treatments.** In the absence of 04-EtTTP during the gap filling reaction, no HincII resistant plasmids were found for any DNA polymerase used (Table 1). However, when 04-EtTTP was the only pyrimidine deoxynucleotide substrate in the polymerase buffer, the percentage of endonuclease resistant clones increased dramatically (Table 1; Plate IV).

The percentages of mutant transformants obtained for AMV DNA polymerase, *M. luteus* DNA polymerase, and *E. coli* DNA polymerase were 47%, 36%, and 26%, respectively. No BamHI resistant clones were found in any sample tested from these groups of mutants. However, PstI resistant plasmids were found in increased numbers. In a sample of 10 randomly chosen mutant transformants from each DNA polymerase treated group, the AMV polymerase plasmids yielded 4 PstI resistant samples, the *M. luteus* DNA polymerase treated group yielded 2 resistant isolates, none were found in the *E. coli* DNA polymerase repaired plasmids.

In order to deduce the nature of the mutational change associated with the generation of HincII resistance, the mutants were screened for TagI sensitivity. TagI recognizes the internal 4 nucleotide sequence (5'-TCGA-3') of the HincII site in pUC8. All of the mutants screened were TagI sensitive.
Plate IV. *Hin*cll resistant mutants obtained from gap misrepair by AMV DNA polymerase in the presence of 100 uM O\textsuperscript{4}-EtTTP as the sole pyrimidine substrate. Lanes A, C, E, G, I and K contain 200 ng pUC8 controls in TE Buffer. Wells B, D, F, H, K and L contain 200 ng pUC8 treated with 1 Unit *Hin*cll in the appropriate Endo.R. buffer. Samples D, F, J, and L which retain form I configuration (S.C.) are Endo.R. resistant mutants. Lanes A and H contain representative wild type *Hin*cll sensitive plasmid and are converted to form III (L.).
Plate V. A comparison of PstI sensitive and resistant pUC8 isolates. Lanes A and C contain 200 ng pUC8 in TE buffer. Lanes B and D are corresponding samples which have been treated with PstI. The sample comprising lanes A and B is a representative PstI sensitive isolate, and is converted to form III (L.) by the activity of this enzyme. The sample in lanes C and D is resistant to the endonuclease activity of PstI and retains the form I (S.C.) state.
Plate VI. Randomly chosen *SalI* resistant pUC8 isolates. Lanes A, C, E, G, and I contain 200 ng untreated pUC8 in TE buffer. Lanes B, D, F, H, and J contain 200 ng pUC8 treated with 200 ng *SalI*. Only the sample in B is converted to form III (L.) which is representative of the wild-type plasmid. The remaining samples remain as form I (S.C.) after treatment, which demonstrates resistance to the endonuclease activity of *SalI*. These resistant isolates are *SalI* mutants.
Equimolar concentrations: SalI Gap Misrepaired pUC8. The SalI gapped and repaired samples also show a mutagenic frequency associated with the 3'-5' exonuclease proofreading capacity of the DNA polymerase used to fill in the gap (Table 1, Plate 5). When equimolar concentrations of pyrimidine deoxynucleotide triphosphates and O4-EtTTP were used in the incorporation mixture, the highest mutation frequency (15%) was found among the transformants from AMV DNA polymerase treated pUC8. SalI resistant isolates occurred at lower frequencies in the *M. luteus* (5%) and the *E. coli* (3%) DNA polymerase treated groups (Table 1).

Controls: SalI Gap Misrepair. In the absence of O4-EtTTP in the polymerase reaction, no SalI resistant plasmids were found in any of the three DNA polymerase treatment groups (Table 1). When O4-EtTTP was the only pyrimidine dNTP present, the mutation frequency at the SalI site increased to levels comparable to or higher than those obtained for the gap misrepaired HindIII groups. The AMV DNA polymerase treated group had the highest percentage of SalI resistant plasmid specimens, 48%. The respective mutational frequencies obtained for the *M. luteus* and *E. coli* DNA polymerase gap misrepaired groups were 37% and 40%. Nearest neighbor analysis disclosed no BamHI or PstI resistant samples.

The SalI resistant clones were tested for HindIII and TagI sensitivity (Plate 7, 8, 9). Of the screened clones, 82% were HindIII resistant; 52% were HindIII, TagI; 13% were HindIII, TagIS; 35% were HindIII, TagIR (Table 5).
Plate VII. Treatment of SalI resistant isolates with HincII. 200 ng of each isolate was treated with SalI (Lanes A, C, E, and G) and HincII (Lanes B, D, F, and H). Each SalI treated isolate retained form I configuration (SC) and are thus resistant mutants. The samples in lanes D, F, and H are sensitive to cleavage by HincII, the sample in lane B is not. This demonstrates that SalI resistant mutants may retain sensitivity to HincII. However, the sample in A and B is a SalI, HincII resistant mutant.
Plate VIII. Restriction endonuclease polymorphism of the shared SalI/TaqI/HincII restriction endonuclease site of pUC8 generated by d4-EtTTP gap misrepair mutagenesis. The samples in lanes A and D were treated with SalI, lanes B and E were treated with TaqI, and lanes C and F were treated with HincII. The sample shown in A, B, and C is SalI and HincII resistant, and TaqI sensitive. The sample in lanes D, E, and F is SalI resistant but is HincII and TaqI sensitive.
Plate IX. Restriction endonuclease polymorphism of the shared SalI/TaqI/HinCII restriction endonuclease site of pUC8 generated by 04-EtTTP gap misrepair mutagenesis. The samples in lanes A and D were treated with SalI, lanes B and E were treated with TaqI, and lanes C and F were treated with HinCII. The sample shown in A, B, and C is SalI, HinCII, and TaqI sensitive. The sample in lanes D, E, and F is SalI, HinCII, and TaqI resistant.
THE EFFECT OF PYRIMIDINE DEOXYRIBONUCLEOTIDE CONCENTRATIONS ON THE
MUTAGENIC EFFICIENCY OF O4-ETHYLTHYMIDINE MISINCORPORATION

The protocol of the initial gap misrepair mutagenesis study was expanded to ascertain the effect of the relative concentrations of pyrimidine deoxynucleotide substrates on the mutagenic efficiency of In vitro O4-EtTTP Incorporation. The results are summarized in Table 2.

The Effect of O4-EtTTP Concentrations

In the misrepair mutagenesis of pUC8 gapped at the HincII restriction endonuclease site, the effects of increasing O4-EtTTP concentrations ranging from 0 to 100 uM in the presence of 100 uM dCTP and TTP were small. The highest number of mutant transformants (5%) were observed with AMV DNA polymerase mediated gap misrepair at 100 uM O4-EtTTP. M. luteus DNA polymerase and E. coli DNA polymerase gap misrepaired plasmid produced 3% and 1% mutant transformants at a 100 uM O4-EtTTP concentration (Table 2).

The Effect of TTP Concentrations

The molar concentrations of dCTP and O4-EtTTP were constant at 100 uM, and the concentration of TTP was increased in ten fold increments from 0 to 100 uM. This treatment regimen did not yield any noteworthy changes in the frequency of occurrence of HincII resistant plasmids in the absence of TTP compared to that in the presence of TTP. However, the mutational frequencies increased slightly with increasing TTP concentrations (Table 2).
Table 2

Effect of Pyrimidine Deoxyribonucleotide Pool Concentrations on O4-EtTTP Misincorporation Mutagenesis

<table>
<thead>
<tr>
<th>TTP, a.</th>
<th>dCTP, b.</th>
<th>TTP, c.</th>
<th>O4-EtTTP d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>dCTP; 0</td>
<td>04-EtTTP; 0</td>
<td>04-EtTTP; -dCTP, +[O4-EtTTP] (µM),</td>
<td>+[TTP] (µM), +[dCTP] (µM), -TTP</td>
</tr>
<tr>
<td>0 1 10 100</td>
<td>0 1 10</td>
<td>0 0 0 1 0 1</td>
<td></td>
</tr>
</tbody>
</table>

HindIII site

<table>
<thead>
<tr>
<th></th>
<th>E. coli pol.</th>
<th>M. luteus pol.</th>
<th>AMV pol.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 0 1 1</td>
<td>0 0 2 3</td>
<td>0 4 3 5</td>
</tr>
<tr>
<td></td>
<td>0 2 2 4 0 2</td>
<td>2 3 3 8 4 5 0</td>
<td>3 3 5 4 4 3 0 4 7</td>
</tr>
<tr>
<td></td>
<td>24 20 15 26</td>
<td>38 34 23 36  2</td>
<td>45 44 30 47</td>
</tr>
</tbody>
</table>

SalI site

<table>
<thead>
<tr>
<th></th>
<th>E. coli pol.</th>
<th>M. luteus pol.</th>
<th>AMV pol.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 0 1 3</td>
<td>0 1 2 5</td>
<td>0 4 6 15</td>
</tr>
<tr>
<td></td>
<td>15 14 10 38</td>
<td>20 18 13 42 5</td>
<td>26 22 15 46 40 38 48</td>
</tr>
<tr>
<td></td>
<td>38 31 25 37</td>
<td>42 35 30 40  2</td>
<td></td>
</tr>
</tbody>
</table>

a. DNA polymerase reaction contained 100 uM dATP, dGTP, dCTP, and TTP, each. O4-EtTTP was added in the concentrations indicated.
b. DNA polymerase reaction contained 100 uM dATP, dGTP, dCTP, and O4-EtTTP, each. TTP was added in the concentrations indicated.
c. DNA polymerase reaction contained 100 uM dATP, dGTP, TTP, and O4-EtTTP, each. dCTP was added in the concentrations indicated.
d. DNA polymerase reaction contained 100 uM dATP, dGTP, and O4-EtTTP, each. dCTP and TTP were not included.
e. 100 plasmid isolates were screened in each sample shown.
The Effect of dCTP Concentrations

The greatest increases in the percentage of HincII resistant plasmids occurred in the protocol where dCTP concentrations were varied from 0 to 100 uM and the TTP and O4-EtTTP concentrations were maintained at 100 uM each (Table 2). In this set of experiments, the mutational frequencies increased from a baseline of 24% generated by E. coli repaired pUC8 in the absence of dCTP, to 45% when the AMV DNA polymerase was the enzyme of choice under the same conditions. As the concentration of dCTP was increased to 100 uM, the mutational frequency decreased, however, the most drastic decline in mutational frequency occurred between the 10 uM and 100 uM concentrations.

Manipulation of the Pyrimidine Deoxynucleotide Triphosphate Concentrations in the Gap Repair of the SalI Site. Gap misrepair of the SalI site by varying pyrimidine concentrations followed the same protocols as described for the gap repair of the HincII restriction endonuclease site. In the first treatment the concentrations of O4-EtTTP were varied in respect to constant TTP and dCTP concentrations. The frequencies of SalI resistant isolates increased as a function of increasing O4-EtTTP concentration. This effect was most pronounced in the AMV DNA polymerase treatment group, followed by isolates from M. luteus DNA polymerase treated plasmids, and finally by E. coli DNA polymerase repaired specimens (Table 2).

The mutagenic efficiencies for SalI resistance resulting from the variation of TTP concentrations were greater than the frequencies observed for HincII gap misrepair under the same conditions. Plasmids obtained from transformants produced by AMV DNA polymerase treated
SalI gapped pUC8 had the highest mutational frequencies in each treatment. In the total absence of dCTP, a 46% SalI resistance frequency was observed. This value decreased to 40% and then to 38% as the concentration of dCTP was increased to 1 uM and 10 uM. As stated before, no mutants were observed with 100 uM dCTP concentrations in this treatment group (Table 2).

Transformants isolated from the M. luteus DNA polymerase misincorporation study demonstrated decreasing SalI resistance mutational frequencies as a function of increased dCTP concentration: 42% at 0 uM dCTP, 35% at 1 uM, 30% at 10 uM, 0 at 100 uM. A similar trend was observed for the E. coli DNA polymerase Large Fragment repaired treatment. These values correspond to the various dCTP concentrations stated above; 38%, 31%, 25%, 0 (Table 2).

The SalI resistance values for misinsertion of O4-EtTTP in the absence of TTP and dCTP have already been stated and were included in Table 1 and Table 2 for comparison.

Restriction enzyme polymorphism typing and analysis of neighboring restriction endonuclease sites were not done in the various studies with altered pyrimidine concentrations.

THE EFFECT OF E. coli ALKYLTRANSFERASE ON THE MUTAGENIC EFFICIENCY OF O4-ETHYLTHYMIDINE MISINCORPORATION

E. coli alkyltransferase was applied to post gap repaired pUC8 by HincII and SalI in order to determine the effect of alkyltransferase on O4-EtTTP misincorporation within these sites and the subsequent effect upon mutagenesis. In Table 3 the effects of this treatment may be seen. When only O4-EtTTP was present as a pyrimidine
deoxynucleotide substrate, AMV DNA polymerase gap misrepair yielded 47% mutation frequencies for \textit{HincII} gap misrepair mutagenesis. Alkylation transferase treatment of plasmid from this protocol yielded a 49% mutation frequency. Similar results were observed for \textit{M. luteus} and \textit{E. coli} DNA polymerase repaired pUC8 transformants. The alkylation transferase treatment of the \textit{M. luteus} DNA polymerase mutagenesis protocol generated a mutation frequency of 39%, and the \textit{E. coli} DNA polymerase repaired plasmids yielded a 28% mutation frequency compared to a 26% occurrence in nonalkylation transferase treated controls.

Among the \textit{SalI} resistance frequencies, a drop in mutagenic efficiency was observed in each polymerase misrepair group. AMV DNA polymerase misincorporation mutagenesis efficiencies decreased from 48 to 39%; \textit{M. luteus} mutagenesis frequencies dropped from 40 to 32%; \textit{E. coli} DNA polymerase mutational rates decreased to 25% from 37% (Table 3).

\textbf{THE MUTATIONAL EFFICIENCY OF T\textsubscript{4} DNA POLYMERASE 3'-5' EXONUCLEASE ACTIVITY IN O\textsuperscript{4}-ETHYLTHYMINE MISINCORPORATION}

\textit{T\textsubscript{4}} DNA polymerase 3'-5' exonuclease misincorporation in \textit{HincII} nicked pUC8 yielded 14% \textit{HincII} resistant plasmid isolates, and 46% \textit{SalI} resistant isolates.

Alkylation transferase treatment of the misincorporated DNA abrogated \textit{HincII} resistance, but increased \textit{SalI} resistance to 50%. However, 10 \textmu M dCTP reduced the original mutational frequencies for \textit{SalI} resistance by the exchange reaction to 12%. The addition of 10 \textmu M TTP increased the mutagenic efficiency of the exchange reaction to 48%.
Table 3

The Effect of *E. coli* Alkyltransferase on the Mutation Frequency of Gap-Misrepaired pUC8

<table>
<thead>
<tr>
<th>HindIII site b.</th>
<th>- Alkyltransferase</th>
<th>+Alkyltransferase</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> pol.</td>
<td>26</td>
<td>28</td>
</tr>
<tr>
<td><em>M. luteus</em> pol.</td>
<td>36</td>
<td>39</td>
</tr>
<tr>
<td>AMV pol.</td>
<td>47</td>
<td>49</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SalI site b.</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> pol.</td>
<td>37</td>
<td>25</td>
</tr>
<tr>
<td><em>M. luteus</em> pol.</td>
<td>40</td>
<td>32</td>
</tr>
<tr>
<td>AMV pol.</td>
<td>48</td>
<td>39</td>
</tr>
</tbody>
</table>

a. 100 plasmid isolates were screened in each sample shown
b. DNA polymerase reaction contained dATP, dGTP, and O^\text{4}-EtTTP, 100 uM each. No dCTP or TTP were added.
Treatment with alkyltransferase increased both values to 15% and 50%, respectively. Inclusion of 100 μM TTP or dCTP eliminated the production of mutants (Table 4).
Table 4

O4-Ethylthymidine Triphosphate Misincorporation Into HindII Nicked pUC8 Via T4 DNA Polymerase 3'-5' Exonuclease Exchange Reaction

<table>
<thead>
<tr>
<th>% HindII resistant Isolates</th>
<th>Percent of AmpR transformants yielding Endo.R, resistant pUC8b*</th>
<th>- Alkyltransferase</th>
<th>+Alkyltransferase</th>
</tr>
</thead>
<tbody>
<tr>
<td>100uM O4E+TTP</td>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>100uM O4E+TTP, 100uM dCTP</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>100uM O4E+TTP, 100uM TTP</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>100uM O4E+TTP, 10uM dCTP</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>100uM O4E+TTP, 10uM TTP</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

% SalI resistant Isolates

| 100uM O4E+TTP              | 46                                                            | 50                |
| 100uM O4E+TTP, 100uM dCTP  | 0                                                             | 0                 |
| 100uM O4E+TTP, 100uM TTP   | 0                                                             | 0                 |
| 100uM O4E+TTP, 10uM dCTP   | 12                                                            | 15                |
| 100uM O4E+TTP, 10uM TTP    | 48                                                            | 50                |

a. 100 plasmid isolates were screened in each sample shown.
<table>
<thead>
<tr>
<th>dNTPs in Reaction</th>
<th>Total Transformants</th>
<th>Transformation Efficiency</th>
<th>Total Colonies Screened</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(transformants/ µg DNA)</td>
<td></td>
</tr>
<tr>
<td>100 µM dCTP, 100 µM TTP, [04-EtTTP] (µM)</td>
<td>256</td>
<td>1.28 x 10^5</td>
<td>100</td>
</tr>
<tr>
<td>0</td>
<td>256</td>
<td>1.28 x 10^5</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>310</td>
<td>1.55 x 10^5</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>178</td>
<td>8.90 x 10^4</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>325</td>
<td>1.63 x 10^5</td>
<td>100</td>
</tr>
<tr>
<td>100 µM dCTP, 100 µM O4-EtTTP, [TTP] (µM)</td>
<td>195</td>
<td>9.75 x 10^4</td>
<td>100</td>
</tr>
<tr>
<td>0</td>
<td>195</td>
<td>9.75 x 10^4</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>121</td>
<td>1.21 x 10^4</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>210</td>
<td>1.05 x 10^4</td>
<td>100</td>
</tr>
<tr>
<td>100 µM TTP, 100 µM O4-EtTTP, [dCTP] (µM)</td>
<td>365</td>
<td>1.83 x 10^5</td>
<td>100</td>
</tr>
<tr>
<td>0</td>
<td>365</td>
<td>1.83 x 10^5</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>480</td>
<td>2.40 x 10^5</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>318</td>
<td>1.59 x 10^5</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 5 (continued)

<table>
<thead>
<tr>
<th>dNTPs in Reaction</th>
<th>Total Transformants</th>
<th>Transformation Efficiency (transformants/μg DNA)</th>
<th>Total Colonies Screened</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 μM O^-E+TTP</td>
<td>345</td>
<td>1.50 x 10^5</td>
<td>100</td>
</tr>
<tr>
<td>- dCTP, -TTP</td>
<td>286</td>
<td>1.43 x 10^5</td>
<td>100</td>
</tr>
<tr>
<td>(+ alkyltransferase)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(- alkyltransferase)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**M. luteus** DNA Polymerase Protocol

<table>
<thead>
<tr>
<th>[dCTP]</th>
<th>[TTP]</th>
<th>[dCTP]</th>
<th>[TTP]</th>
<th>[dCTP]</th>
<th>[TTP]</th>
</tr>
</thead>
<tbody>
<tr>
<td>(μM)</td>
<td></td>
<td>(μM)</td>
<td></td>
<td>(μM)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>146</td>
<td>0</td>
<td>345</td>
<td>0</td>
<td>179</td>
</tr>
<tr>
<td>1</td>
<td>147</td>
<td>1</td>
<td>482</td>
<td>1</td>
<td>115</td>
</tr>
<tr>
<td>10</td>
<td>186</td>
<td>10</td>
<td>216</td>
<td>10</td>
<td>184</td>
</tr>
</tbody>
</table>

*Note: Additional columns and rows for other dNTPs and concentrations are not shown in this snippet.*
Table 5 (continued)

<table>
<thead>
<tr>
<th>dNTPs In Reaction</th>
<th>Total Transformants</th>
<th>Transformation Efficiency (transformants/ ug DNA)</th>
<th>Total Colonies Screened</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 uM dCTP, 100 uM O₄-ETTP</td>
<td>197</td>
<td>9.85 x 10⁴</td>
<td>100</td>
</tr>
<tr>
<td>(+ alkyltransferase)</td>
<td>128</td>
<td>6.40 x 10⁴</td>
<td>100</td>
</tr>
</tbody>
</table>

**AMV DNA Polymerase Protocol**

<table>
<thead>
<tr>
<th>dNTPs In Reaction</th>
<th>Total Transformants</th>
<th>Transformation Efficiency (transformants/ ug DNA)</th>
<th>Total Colonies Screened</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 uM dCTP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 uM dCTP, 100 uM O₄-ETTP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[O₄-ETTP] (uM)</td>
<td>0</td>
<td>163</td>
<td>6.50 x 10⁴</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>155</td>
<td>7.75 x 10⁴</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>142</td>
<td>7.10 x 10⁴</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>148</td>
<td>7.40 x 10⁴</td>
</tr>
<tr>
<td>100 uM dCTP, 100 uM O₄-ETTP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[TTP] (uM)</td>
<td>0</td>
<td>136</td>
<td>6.80 x 10⁴</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>129</td>
<td>6.45 x 10⁴</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>144</td>
<td>7.20 x 10⁴</td>
</tr>
<tr>
<td>100 uM TTP, 100 uM O₄-ETTP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[dCTP] (uM)</td>
<td>0</td>
<td>124</td>
<td>6.20 x 10⁴</td>
</tr>
</tbody>
</table>
Table 5 (continued)

<table>
<thead>
<tr>
<th></th>
<th>Total Transformants</th>
<th>Transformation Efficiency (transformants/ µg DNA)</th>
<th>Total Colonies Screened</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>119</td>
<td>$5.95 \times 10^4$</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>115</td>
<td>$5.75 \times 10^4$</td>
<td>100</td>
</tr>
</tbody>
</table>

100 uM $^{32}$P-dTTP

- dCTP, -TTP

(+ alkyltransferase) 116  $5.80 \times 10^4$  100
(- alkyltransferase) 182  $9.10 \times 10^4$  100

T₄ DNA Polymerase Protocol

<table>
<thead>
<tr>
<th>dNTPs in Reaction¹</th>
<th>Total Transformants</th>
<th>Transformation Efficiency (transformants/ µg DNA)</th>
<th>Total Colonies Screened</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 uM $^{32}$P-dTTP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(- alkyltransferase)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-dCTP, -TTP</td>
<td>221</td>
<td>$1.11 \times 10^5$</td>
<td>100</td>
</tr>
<tr>
<td>100 uM dCTP</td>
<td>219</td>
<td>$1.10 \times 10^5$</td>
<td>100</td>
</tr>
<tr>
<td>10 uM dCTP</td>
<td>142</td>
<td>$7.10 \times 10^4$</td>
<td>100</td>
</tr>
<tr>
<td>100 uM TTP</td>
<td>101</td>
<td>$5.05 \times 10^4$</td>
<td>100</td>
</tr>
<tr>
<td>10 uM TTP</td>
<td>224</td>
<td>$1.12 \times 10^5$</td>
<td>100</td>
</tr>
<tr>
<td>(+ alkyltransferase)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-dCTP, -TTP</td>
<td>126</td>
<td>$6.30 \times 10^4$</td>
<td>100</td>
</tr>
<tr>
<td>100 uM dCTP</td>
<td>187</td>
<td>$9.35 \times 10^4$</td>
<td>100</td>
</tr>
<tr>
<td>10 uM dCTP</td>
<td>325</td>
<td>$1.63 \times 10^5$</td>
<td>100</td>
</tr>
<tr>
<td>100 uM TTP</td>
<td>428</td>
<td>$2.14 \times 10^5$</td>
<td>100</td>
</tr>
<tr>
<td>10 uM TTP</td>
<td>241</td>
<td>$1.22 \times 10^5$</td>
<td>100</td>
</tr>
</tbody>
</table>

a. Reaction also includes 100 µM dATP, and 100 µM dGTP.
b. Reaction does not include 100 µM dATP, and 100 µM dGTP.
CHAPTER IV
DISCUSSION

The mutagenic effects of $O^4$-EtTTP misincorporation in the $HindIII$ and $SalI$ gap misrepair mutagenesis protocols described in this investigation are quite pronounced and result in a rather high level of mutagenicity. The basis for this claim rests on the evidence provided by the experimental design of this investigation.

THE INTERACTION OF PROOFREADING ABILITY WITH MISINCORPORATION MUTAGENIC EFFICIENCY

The first set of experimental results, shown in Table 1, reflect the influence of DNA polymerase proofreading function on the mutagenic efficiency of $O^4$-ethylthymine misincorporation in gap misrepair. In both the $HindIII$ and $SalI$ gap repair protocols, the polymerase enzymes used differed mainly in their 3'-5' exonuclease repair activity.

Effects of proofreading activity on mutational efficiency have been shown for DNA polymerase mediated misinsertion of noncomplementary bases by primer elongation. Primer elongation was used to generate revertant phenotypes by the misincorporation of a noncomplementary base at the $am\_18$ and $am\_3$ loci of $\phi X174$. On the basis of the mutagenic efficiencies reported in these studies, AMV DNA polymerase has a lower fidelity of DNA synthesis than $T_4$ DNA polymerase (Zakour, James and Loeb, 1984). Primer extension by AMV
DNA polymerase generated higher reversion frequencies than T4 DNA polymerase extended primers. The higher reversion frequency is indicative of a higher level of base misincorporation. Comparable results have been shown for E. coli DNA polymerase and T4 DNA polymerase. E. coli DNA polymerase appears to have lower fidelity than T4 DNA polymerase, but higher than AMV DNA polymerase (Zakour and Loeb, 1982; Zakour, James, and Loeb, 1984). In this study the mutagenic efficiency of O^4-ethylthymine misincorporation into gapped restriction endonuclease sites reflects the fidelity of the DNA polymerases in a manner like the above studies.

Gap repair in the absence of O^4-EtTTP did not produce any restriction endonuclease resistant plasmid isolates (Table 1). This is indicative of normal 5'-3' polymerase activity in the presence of optimal and balanced concentrations of the normal deoxynucleotide triphosphates. However, when the molar content of O^4-EtTTP in the incorporation reaction was equal to the concentrations of dCTP and TTP, the mutagenic frequency increased to a small extent. In the case of HincII gap misrepair, the mutagenic frequency did not exceed a maximum of 5%. This maximum was generated by AMV DNA polymerase at 100 uM concentrations each of O^4-EtTTP, dCTP, and TTP (Table 1). The same reaction conditions in the SalI gap misrepair mutagenesis study gave a 3-fold higher mutational frequency. This increase may be attributed to the 3-fold greater number of pyrimidine sites available for mutagenesis in the SalI restriction endonuclease site (1 thymine and 2 cytosine) (Figure 6). These data represent baseline effects that indicate DNA polymerase specificity for the normally occurring
bases. Previously described mutagenesis assays show baseline mutation frequencies which are comparable to those observed in Table 1 (Shortle and Botstein, 1983; Ripley and Shoemaker, 1983). Compared to AMV DNA polymerase, lower mutation frequencies were observed for *M. luteus* DNA polymerase gap misrepaired pUC8 transformants in both the *Hinf*II and *Sall* sites. The frequencies for *E. coli* DNA polymerase repaired pUC8 transformants was the lowest observed in this group. The proofreading ability of these polymerases appears to be having an effect on these values, since it is the only observable difference in the reaction conditions. These observations reflect the effects of proofreading activity observed by others (Kunkel, et al., 1981; Zakour, James, and Loeb, 1984). Thus, DNA polymerase proofreading activity plays a role in the prevention of base misincorporation in DNA by gap misrepair. The proofreading capacity of the DNA polymerases was an influential factor in the mutagenic efficiency of *O*-EtTTP misincorporation. AMV DNA polymerase gap misrepaired DNA yielded the highest number of mutants, followed by *M. luteus* and then *E. coli* DNA polymerase filled gaps.

When the normal pyrimidine deoxyribonucleotide triphosphates were absent and *O*-EtTTP was present at optimal levels during gap misrepair, the mutational frequencies of each of these DNA polymerase treatment group increased substantially. In the absence of 3'-5' exonuclease proofreading ability, the mutational frequencies obtained from AMV DNA polymerase treated pUC8 were very close to the theoretical mutational frequency. Recent reports have also stated that AMV DNA polymerase may yield similarly high mutational
frequencies in primer elongation mutagenesis of ØX174 and M13mp2 (Zakour, James, and Loeb, 1984). In the most efficient of mutagenic strategies, an unrepaired base misincorporation in DNA base would yield a 50% mutational frequency (Shortle and Nathans, 1981). This is because during semiconservative DNA replication the two DNA strands comprising the plasmid would separate and yield two templates. One, the gap misincorporated strand would provide a template containing an erroneous base resulting in the synthesis of a daughter strand having a base complementary to the misinserted base. The other strand, which remained intact during the gapping and DNA polymerase repair process would retain the wild type sequence because the mutagenesis treatments had not affected this strand at all.

Bearing in mind the fact that DNA polymerase proofreading activity varies among the DNA polymerases used in the in vitro gap misrepair schemes, it appears that in each case where O4-EtTTP is included in the polymerase reaction, the mutation frequency increases as a function of decreasing proofreading activity. Also, the number of potential sites available for misincorporation influence the mutagenic frequency associated with the O4-ethylthymine incorporation. This is because of the increased probability of O4-ethylthymine incorporation as a function of increased available sites.

It is also important to note that in the absence of dCTP and TTP the mutational frequencies for gap misrepair mutagenesis are substantially higher than for the cases where these dNTP's are present. This is indicative of the utilization of O4-EtTTP instead of dCTP or TTP. In vitro polymerase reactions have shown that
$O^4$-alkylthymidine triphosphate is a suitable substrate for these enzymes, and is incorporated by polymerases against either adenine or guanine (Singer, Sagl, and Kusmierek, 1983; Singer, Abbott, and Spengler, 1984). The mutagenic efficiency of $O^4$-EtTTP in gap-misrepair mutagenesis is not only influenced by polymerase fidelity but also by the presence of the normal deoxynucleotides. The inclusion of the normal bases at 100 µM concentrations almost eliminates the mutagenic effects of an equal concentration of $O^4$-ethylthymidine triphosphate. However, in the absence of these nucleotides, the polymerases use $O^4$-ethylthymidine triphosphate as a pyrimidine substrate incorporating as thymine or cytosine. This evidence indicates that TTP and dCTP compete with $O^4$-EtTTP during polymerase reactions.

The effect of the absence or presence of TTP and dCTP in the gap misrepair reaction mixture arises from an interaction between the pyrimidine dNTP's, the substrate specificities of the DNA polymerases and their proofreading capacities. Polynucleotide templates containing $O^4$-methylthymine direct the misincorporation of guanine by polymerases. However, the number of guanine misincorporations increases when error prone AMV DNA polymerase is used (Singer, Abbott, and Spengler, 1984) (Figure 6). $O^4$-ethylthymine is incorporated as an alkylated deoxynucleotide by enzymes capable of excising mismatched bases. Thus, the probability of misincorporation mutagenesis will increase during \textit{in vivo} DNA synthesis (Singer, Sagl, and Kusmierek, 1983).
The manipulation of pyrimidine dNTP concentrations in regard to O⁴-EtTTP concentrations during gap repair appears to be a relevant consideration. There are indications that misincorporation by a polymerase can be influenced by the substrate concentrations of the reaction mixture. In primer elongation mutagenesis, the manipulation of deoxynucleotide concentrations in the reaction mixture had a profound influence on the mutagenic efficiency of polymerase misincorporation (Zakour, James, and Loeb, 1984). The error rate of a polymerase is proportional to the ratio of complementary and noncomplementary nucleotides in the reaction mixture (Loeb and Kunkel, 1982).

THE EFFECTS OF NUCLEOTIDE POOL CONCENTRATIONS ON THE EFFICIENCY OF GAP DIRECTED MISINCORPORATION MUTAGENESIS

Alkylation of the nucleotide pool may be an important aspect of mutagenesis. A significant amount of base alkylation has been found to occur in the cellular deoxynucleotide pool and may function as a source of potential alkylated base misincorporations (Topal and Baker, 1982). Much attention has gone into the study of O⁶-methyldeoxyguanosine triphosphate as a precursor deoxynucleotide. The ambiguous hydrogen bonding behavior of O⁶-alkylguanine makes it possible to be misincorporated with thymine during DNA synthesis. In fact, misincorporation of O⁶-methylguanine with thymine occurs preferentially to the formation of O⁶-methylguanine-cytosine pairs (Toorchen and Topal, 1983). O⁶-methyldeoxyguanosine triphosphate also competes with dATP for incorporation against thymine.
Figure 7. The base pairing between $\text{O}^4$-ethylthymine and adenine; and between $\text{O}^4$-ethylthymine and guanine.
Thus, nucleotide pool interactions are also an important consideration in mutagenesis. In the previous section, competitive activity was also observed between $O^4$-EtTTP and the normal pyrimidine deoxynucleotide triphosphates.

The effects of the pyrimidine dNTP interactions with DNA polymerase substrate specificity and proofreading ability were addressed by manipulating the concentrations of the normal pyrimidine substrates and $O^4$-EtTTP with regard to one another. In this way the relevant nucleotide pool effects on the mutagenic effects of $O^4$-EtTTP incorporation could be ascertained.

In the first treatment, concentrations of the normal pyrimidines and purines were kept uniform at 100 uM, an optimal concentration for DNA polymerase reactions (Shortle and Botstein, 1983). The concentration of $O^4$-EtTTP was increased in tenfold increments from 0 to 100 uM in each DNA polymerase directed synthesis. For each of these treatments only a slight mutagenic response was observed.

The mutagenic frequency of DNA polymerase directed *SalI* gap repair of pUC8 shows an increase which reflects the three fold greater number of pyrimidine loci above the *HinCII* gap repaired pUC8. The ability of the DNA polymerase used to correct a misinsertion via 3'-5' exonuclease activity is evident in the differences between the results yielded by the various polymerases. Contrast, for example, the 15% frequency observed with AMV polymerase directed gap misrepair of *SalI* with the 3% value obtained with *M. luteus* DNA polymerase directed gap misrepair at 100 uM concentrations of each pyrimidine substrate used (Table 2). However, the mutation frequency increased in a
concentration dependent fashion as the relative availability of
$O^4$-EtTTP was increased (Table 2). This increase is small and almost
negligible in the \textit{Hin}cII gap repaired plasmids. This is because of
the availability of only 1 site for $O^4$-ethylthymine misincorporation,
the sixth base of the sequence, 5'-GTCGAC-3'. The error prone AMV DNA
polymerase repaired plasmids yielded a higher proportion of
restriction enzyme resistant isolates compared to \textit{E. coli} gap repaired
plasmids. This effect increased as the concentration of $O^4$-EtTTP
increased in the reaction mixture (Table 2).

The effects of TTP concentrations in the mutational efficiency of
$O^4$-ethylthymine gap repair misincorporation mutagenesis was the next
permutation investigated. The results of this study indicate a
dramatic difference between the \textit{Hin}cII and \textit{Sal}I sites (Table 2). In
the \textit{Hin}cII gap repaired pUC8 plasmids, the effect of TTP in the
polymerase buffer was small, and not substantially different from the
effects observed in the first treatment in which dCTP, TTP, and
$O^4$-EtTTP were present in 100 \textmu M concentrations. This effect is most
likely due to the fact that the only available site in the \textit{Hin}cII
gapped region is a cytosine residue and that the presence of an
optimal concentration of cytosine is available under these reaction
conditions. The absence of TTP would not be expected to affect the
integrity of the cytosine site involved. Thus, the mutation frequency
generated in the \textit{Hin}cII gap repaired plasmids reflect the conditions
of equimolar concentrations of TTP, dCTP, and $O^4$-EtTTP.
Among the SalI gap misrepair mutagenesis transformants, the effect of TTP concentrations had a profound effect on the mutation frequency. In the absence of TTP, the mutational frequency of plasmid isolates is highest among the AMV DNA polymerase repaired transformants. This mutational frequency decreases as a function of proofreading activity and TTP concentration. Ultimately, the occurrence of SalI resistance is overcome by the equimolar concentrations of TTP. This effect is best explained by the presence of a thymine residue available at the second position of the SalI recognition sequence. Thus, it appears that thymine competes with O4-ethylthymine for that position, and that the mutational effects seen here are almost solely the result of O4-ethylthymine misincorporation at the second nucleotide. This is more clearly understood when comparison with the effects of TTP concentrations on HincII mutagenesis is made. Cytosine residues are not really affected by TTP concentrations and it is almost obvious that the effects observed with SalI gap misrepair under varying TTP concentrations is due to the available thymine site.

The effect of dCTP concentrations on the SalI resistance mutational frequency of the gap misrepaired pUC8 transformants was greatest in response to all the polymerases used. In the absence of dCTP, the yield of SalI resistant plasmid isolates closely approximates the percentages obtained when this restriction site was repaired by DNA polymerases in the presence of only O4-EtTTP. This effect declined as a function of increasing dCTP concentration and proofreading ability, as has been seen in the other dNTP permutations.
Nevertheless, the effect of dCTP exerts the greatest effect in the efficiency of $O^4$-ethylthymidine incorporation at the *Sal*I site. These results indicate an ability of the DNA polymerases to utilize $O^4$-EtTTP as a substrate in repair synthesis. The difference between the results obtained by varying dCTP concentrations are almost indistinguishable from those obtained when $O^4$-EtTTP is the only pyrimidine deoxynucleotid available (Table 1). The mutational frequencies observed here reflect base changes at the cytosine residues of this site. This conclusion is based on the observation that varying TTP concentrations do not affect dCTP sites. TTP is present in this reaction at levels which abolished the mutagenic effects of $O^4$-EtTTP in *Sal*I due to low TTP concentrations.

On the basis of the evidence given here, $O^4$-EtTTP misincorporation causes the greatest mutational effects when incorporated in place of cytosine. The availability of a thymine site in the *Sal*I site also has some effect in increasing the mutational frequencies of the misincorporation strategies, yet these effects are not as pronounced as those observed for the interaction of cytosine. Given the hypothesis that $O^4$-ethylthymine can base pair efficiently with guanine by forming thermodynamically stable hydrogen bonds at the $O^2$-, and N-3 positions of the pyrimidine ring, the experimental evidence shown here demonstrates that $O^4$-ethylthymine behaves similarly to cytosine and is readily incorporated instead. This is consistent with the observations that $O^4$-alkylthymines can form complementary base pairs with guanine during DNA synthesis (Singer, Sagl, and Kusmlerek, 1983).
In comparison, the mutagenic behavior of $O^4$-ethylthymine parallels the characteristics observed for $O^6$-methylguanine in vitro as a nucleotide pool substrate. In vitro DNA synthesis of $T_7$ amber mutants in the presence of $O^6$-methyldeoxyguanosine triphosphate resulted in the production of revertant isolates (Dodson, et al., 1982). The results of this study indicate that the misincorporation of $O^6$-methylguanine across from thymine may also occur as an effect of deoxynucleotide pool.

**THE EFFECT OF IN VITRO E. coli ALKYLTRANSFERASE TREATMENT ON THE MUTAGENICITY OF $O^4$-ETHYLTHYMIDINE GAP MISREPAIR**

The role of alkyltransferase in the repair of alkylated base damage in DNA has been implicated as a mutagenic process (Dodson, et al., 1982; Toorchen and Topal, 1983). For example, the repair of an $O^6$-methylguanine-thymine base pair would generate a guanine-thymine mispair which gives A-T to G-C transition mutations. A similar case has been demonstrated for the alkyltransferase mediated repair of $O^4$-ethylthymine-guanine pairs. Alkyltransferase repair causes the formation of a thymine-guanine mispair, which in turn generates a C-G to T-A transition mutation. The importance of alkyltransferase repair was illustrated in an experimental system using $O^6$-methylguanine inserted into the PstI site of phage M13mp8. When this phage was transfected into E. coli which had been treated with MNNG the frequency of G-C to A-T transition mutations increased dramatically above untreated controls (Loechler, Green, and Essigman, 1984). During in vitro synthesis of phage $T_7$ DNA, the presence of $O^6$-methyldeoxyguanosine increased the mutation frequency above
background (Dodson, et al., 1982). Part of the mutational effects in this system were believed to arise from the preferential mispairing of O\textsuperscript{6}-methylguanine with thymine residues, which result in a transition mutation when alkyltransferase removes the methyl group.

Clarification of the role of base alkylation repair in the mutagenic effects of O\textsuperscript{4}-ethylthymine incorporation in the Hinc\textsubscript{II} and Sal\textsubscript{I} sites was provided by treating a sample O\textsuperscript{4}-EtTTP Incorporated plasmids with E. coli alkyltransferase prior to transformation of JM 83. The Hinc\textsubscript{II} gap misrepaired transformants did not show an increase in mutational frequency when treated with alkyltransferase. The Sal\textsubscript{I} gap misrepaired transformants showed a decreased mutational frequency when treated with alkyltransferase. The decrease was 12\%, 8\%, and 9\% for gap repair by E. coli, M. luteus, and AMV DNA polymerases, respectively. These observations shed a new insight into the mutational effect of O\textsuperscript{4}-ethylthymine in DNA. When incorporated across from guanine, alkyltransferase repair only serves to fix the misincorporation as a mutational event. That goes to say, that during the next round of replication, the Incorporation of guanine across from a template of O\textsuperscript{4}-ethylthymine is not possible. Thus, when an O\textsuperscript{4}-ethylthymine-guanine pair is repaired by alkyltransferase, the result of replication in the next round is to yield one T-A pair and one C-G pair. The decreased mutagenic efficiencies in the Sal\textsubscript{I} gap misrepaired alkyltransferase treatment groups illustrates the effect of O\textsuperscript{4}-ethylthymine misincorporation at the thymine position of the Sal\textsubscript{I} site. When an O\textsuperscript{4}-ethylthymine-adenine pair is repaired by alkyltransferase, the mutational activity of that misincorporation is
abolished. The base pair returns to the normal configuration. This effect has been demonstrated with O\(^6\)-methylguanine-cytosine base pairs. The action of alkyltransferase abolishes the potential mutagenicity of O\(^6\)-methylguanine (Loechler, Greene, and Essigman, 1984).

If O\(^4\)-ethylthymine is not repaired, the potential for O\(^4\)-ethylthymine to direct guanine misincorporation exists (Singer, Abbott, and Spengler, 1984). This may cause a T-A to C-G transition mutation at the second base of the SalI site. The residual mutation frequency in the SalI site may be a reflection of the fixing of a T-G mismatch at the third and sixth base positions of the SalI site. In this case, 2 guanine residues in the template DNA strand are available in the gap for mispairing with O\(^4\)-ethylthymine. Consequently, the alkyltransferase treatment of an O\(^4\)-ethylthymidine-guanine misincorporation at these positions favors the fixation of this lesion at this site by generating a T-G base pair. This effect is seen in the Table 3.

**T\(_4\) DNA POLYMERASE 3'-5' EXONUCLEASE ACTIVITY AS A VEHICLE FOR O\(^4\)-ETHYLTHYMIDINE MISMATCHING**

The ability of T\(_4\) DNA polymerase to hydrolyse nicked DNA in a 3'-5' direction from the nick in the absence of dNTPs is well documented (Shortle and Nathans, 1981; Zakour, James, and Loeb, 1984). In the presence of a single dNTP, the 3'-5' exonuclease activity and the 5'-3' polymerase activity of T\(_4\) DNA polymerase establish an equilibrium at the first location of that base at a position 3' to the nick. This mechanism establishes an exchange reaction between the
base in the DNA and the analogous deoxynucleotide triphosphate in the polymerase reaction buffer. The exchange reaction was established at the nick generated between the third and fourth bases in the *Hin*<sub>c</sub><sub>II</sub> sequence. Because the specificities of *Sal*<sub>I</sub> and *Hin*<sub>c</sub><sub>II</sub> for this sequence differ, the plasmid isolates in this section were tested for resistance to both restriction endonucleases. *Hin*<sub>c</sub><sub>II</sub> will cleave this sequence if the third base is either pyrimidine, *Sal*<sub>II</sub> will not. As may be seen in Table 4, 0<sup>4</sup>-EtTTP exchanges in this reaction generates a very high frequency of mutations of *Sal*<sub>II</sub> resistance, an effect which is slightly amplified by treatment with alkyltransferase. However, *Hin*<sub>c</sub><sub>II</sub> resistance was considerably lower. In this test, 14% of the plasmid isolates tested were negative. This *Hin*<sub>c</sub><sub>II</sub> mutation frequency was abrogated by treatment with alkyltransferase. The low frequency of *Hin*<sub>c</sub><sub>II</sub> resistant mutants, and the disappearance of these mutants in the presence of alkyltransferase, dCTP, and TTP indicates that these mutants are being formed at the thymine site in the second base position of the sequence. The high frequency of *Sal*<sub>II</sub> resistant mutants, which in all but 14% of the isolates are *Hin*<sub>c</sub><sub>II</sub> sensitive, shows that the base change that is taking place is predominantly at the third position, or cytosine. This observation is quite revealing in terms of what the 3'-5' proofreading capacity of T<sub>4</sub> DNA polymerase is capable of doing in averting misincorporation of 0<sup>4</sup>-EtTTP into a pyrimidine site. The first point to be made is that 0<sup>4</sup>-EtTTP can behave as cytosine or as thymine in limiting hydrolysis of nicked double stranded DNA, and as an exchange
substrate. This observation is validated by the elimination of the \textit{HindIII} resistance mutation by treatment of the plasmid with alkyltransferase. By implication, the \textit{HindIII} resistance mutation was generated by the incorporation of $O^4$-ethylthymine at the second base site in this sequence to yield an $O^4$-ethylthymine-adenine pair which subsequently produced a T-A to C-G transition. This activity was eliminated when the ethyl group was removed, consequently, no effect was seen at this site. However, the incorporation of $O^4$-ethylthymine at the third base position of \textit{SalI} was not reversed by the alkyltransferase treatment. The extent of $3'-5'$ hydrolysis may be controlled by the presence of $O^4$-ethylthymine acting in an exchange reaction with cytosine. In the presence of an equimolar ratio of dCTP to $O^4$-EtTTP, no \textit{SalI} or \textit{HindIII} resistant plasmids were isolated. When the concentration of dCTP was dropped to 10 uM in the presence of 100 uM $O^4$-EtTTP, the mutational frequency of the \textit{SalI} resistant isolates increased to 12%. Alkyltransferase treatment increased this value to 14%. No \textit{HindIII} resistant isolates were observed. In this context it appears that the majority of the \textit{SalI} mutational effect in the absence of dCTP is attributable to the exchange of $O^4$-EtTTP for cytosine at this third base of the \textit{SalI} sequence. Furthermore, the elimination of \textit{HindIII} resistant isolates from the sample population at 100 uM and 10 uM concentrations of TTP in the $O^4$-EtTTP replacement reaction. The generation of \textit{SalI} mutation frequencies with and without alkyltransferase treatment which are near the theoretical maximum (50%) further indicates that $O^4$-EtTTP is undergoing an exchange reaction with cytidine.
0\textsuperscript{4}-Ethylthymidine is not necessarily being incorporated as result of 3\textsuperscript{'} hydrolysis to the thymine residue at position 2 followed by 5\textsuperscript{'}-3\textsuperscript{'} polymerase insertion in place of the cytidine residue at position 3. The effect of the other nucleotide bases reflects previous work which indicates that substitution mutagenesis is a function of deoxynucleotide pool balance (Sinha and Halmes, 1981).

**THE SIGNIFICANCE OF ALTERED RESTRICTION ENZYME SPECIFICITIES AT THE SHARED HincII and SalI SITE.**

The mutational changes generated in the recognition sequence for SalI and HincII in pUC8 molecule were characterized by the use of the restriction endonucleases SalI, HincII, and TagI. By the use of this strategy the exact location of 100\% of the HincII and 48\% of the SalI mutational changes were localized.

The sequence in question, 5\textsuperscript{'-}GTCCGAC-3\textsuperscript{'}\textsuperscript{,} is recognized by 3 restriction endonucleases. SalI is the most specific for the hexanucleotide sequence, 5\textsuperscript{'-}GTCCGAC-3\textsuperscript{'}\textsuperscript{.} HincII has an ambiguity of specificity pertaining to bases 3 and 4 of the sequence. The third nucleoside may be either thymine or cytosine, and the fourth may be either adenine or guanine. Because of this ambiguity, the HincII recognition sequence may read 5\textsuperscript{'-}GTPPyPuAC-3\textsuperscript{'}\textsuperscript{,} where Py and Pu represent the pyrimidine and purine bases, respectively. TagI, in turn, recognizes the specific four base sequence 5\textsuperscript{'-}TCGA-3\textsuperscript{'} contained within the SalI site. There are two other TagI sites in the pUC8 molecule, thus the characteristic observed for TagI resistance is the disappearance of a normal three band pattern observed in agarose gel electrophoresis, and the appearance of a two band pattern.
The Indicator of SalI and HincII resistance is the failure of the enzyme to convert the DNA from supercoiled form I DNA to linear form III. The distinction between these forms is readily apparent in agarose gel electrophoresis, form I DNA migrates faster than form III. The migration of form III DNA is hindered by the gel matrix; form I is more compact and moves through the gel matrix with less resistance. The novel banding associated with TaqI resistance in pUC8 consists of one of the original bands migrating at a position homologous to the same segment produced by TaqI in the wild-type plasmid. However, a new band appears which represents the TaqI resistant fragment, and migrates at a rate equivalent to the combined molecular weight of the two fragments generated in the normal plasmid.

The HincII resistant isolates were all phenotypically SalI-, HincII-, and TaqI+. This restriction endonuclease pattern indicates that the internal four nucleotides, 5'-TCGA-3' remained intact. Thus, the location of the base change lies external to this region. The novel sequence, 5'-GTCGAT-3', fits this restriction endonuclease polymorphism. Neither HincII nor SalI will recognize and cleave this changed sequence. The BamHI and PstI sensitivity of the majority of these plasmids indicates unaltered adjacent 5' and 3' restriction sites. The O4-E+TTP gap misrepair mutagenesis of the HincII site in pUC8 results in the generation of a single point mutation at the sixth nucleotide of the sequence; a mutation which may correspond to a C-G to T-A transition.
The mutagenesis of the SalI gapped site was somewhat more involved due to the presence of 3 pyrimidine nucleotides in this site. The normal phenotype of the pUC8 plasmid is SalI^S, Hincll^S, TagI^S. Among the plasmids generated by the gap misrepair of the SalI site by AMV DNA polymerase in the presence of 100 μM dGTP, dATP, and 0^-EtTTP each, 52% of the SalI^R isolates were also Hincll^R, and TagI^R. This indicates that a base change may have taken place at the 2 and 3 pyrimidine sites in the hexanucleotide. A change may also have occurred at the 6 cytosine residue. The possible pyrimidine transition mutations which would demonstrate this restriction endonuclease phenotype are as follows: 5'-GTATG-3', base changes at positions 3 and 6; 5'-GCTGAT-3', transitions at nucleosides 2, 3, and 6; 5'-GCGGAT-3', base changes at 2 and 6; 5'-GCTGAC-3', mutation at bases 2 and 3; 5'-GCGGAC-3', mutation at base 2.

Two other restriction endonuclease phenotypes were produced by the AMV gap misrepair of the SalI site of DNA. The most frequently occurring phenotype is SalI^R, Hincll^S, TagI^R. This phenotype implies the sequence 5'-GTTGAC-3', a single base change at the third nucleotide, corresponding to a C to T transition. Finally, 13% of the mutants in this study were, SalI^R, Hincll^R, TagI^S, the same as with the Hincll gap misrepair mutants. This phenotype, as stated before may correspond to the sequence, 5'-GTGAT-3'. This altered site may be the result of a C-G to T-A transition at the terminal base of the SalI site.
SUMMARY

The mutagenic activity of $O^d$-ethylthymine in the gap-misrepair mutagenesis scheme described here is a complex of interactions involving deoxyribonucleotide triphosphate concentrations, DNA polymerase specificities, proofreading activities, alkyltransferase and mismatch-repair effects. The mutagenic frequencies observed in the various polymerase fidelity and deoxynucleotide pool assays of this study are comparable to the those obtained by noncomplementary base misinsertion at the $am18$ locus of $\theta X174$ by primer elongation mutagenesis (Zakour, James, and Loeb, 1984). In this protocol, primer elongation by AMV DNA polymerase was used to misinsert a noncomplementary base in the $am18$ locus to generate revertants. The mutagenic efficiency of this method as reported by the authors was 33%. This highly efficient reversion frequency closely approximates the theoretical maximum of 50%. However, in a recent report (Preston, Loeb, and Singer, 1985), the efficiency of amber mutant reversions caused by the misincorporation of $O^d$-ethylthymidine was not significantly higher than those which occur spontaneously. However, in this study $O^d$-ethylthymine was incorporated across from the adenine residue of the $am3$ locus. Consequently, repair by host alkyltransferase abolished the mutagenic effect of the lesion.

In other mutagenesis studies, deletion loop mutagenesis of the EcoRI site in the Large T antigen gene of an SV40/pBR322 recombinant plasmid by sodium bisulfite deamination of cytosine (Kalderon and Smith, 1984) residues yielded 30 to 50% EcoRI resistance frequencies, an effect which closely approximates the theoretical maximum value.
Gap misrepair mutagenesis of BglI nicked SV40 DNA treated with sodium bisulfite resulted in nearly 50% BglI resistance among the screened isolates (Shortle and Nathans, 1978).

Mutagenesis studies with O\(^6\)-methylguanine have also utilized \textit{in vitro} and site specific approaches. \textit{In vitro} mutagenesis of amber mutant bacteriophage T7 DNA by the inclusion of O\(^6\)-methylguanine during DNA synthesis resulted in the generation of revertants, a result of an A-T to G-C transition mutation. This study represents a very important characterization of alkylated deoxynucleotide pool effect. On the basis of this report, the utilization of O-alkylated nucleotides during DNA synthesis is a mutagenic process. Other studies with \textit{in vitro} DNA replication using \textit{E. coli} polymerase Large Fragment or T\(_4\) DNA polymerase in sequencing reactions (Sanger and Coulson, 1975) further indicated the mutagenic role of O\(^6\)-methylguanine as a deoxynucleotide pool substrate (Topal, Hutchinson, and Baker, 1982; Topal and Toorchen, 1983). The results of these investigations also indicated that O\(^6\)-methylguanine is preferentially misincorporated against thymine, and forms stable hydrogen bonds. In this pair the O\(^2\)- and N-3 positions of thymine hydrogen bond with the N\(^2\) and N-1 positions of O\(^6\)-methylguanine (Topal and Toorchen, 1983). In fact, this base mispairing is the result of the stability of the hydrogen bonding between these bases. The hydrogen bonding conformation between O\(^6\)-methylguanine and thymine is identical to that found between O\(^4\)-ethylthymine and guanine (Figure 6). On the basis of this physical conformation and the known ambiguity of O\(^4\)-alkylthymine base pairing,
$O^4$-ethylthymine preferentially misincorporates against guanine during DNA synthesis. The ability of $O^4$-ethylthymine to form only one hydrogen bond with adenine further suggests preferred base pairing with guanine (Figure 6).

In a forward mutation assay $O^6$-methyldeoxyguanosine triphosphate was incorporated into the beta-lactamase gene of bacteriophage R208 (Eadle, et al., 1984). Transformed bacteria were screened for ampicillin susceptibility, an indication of a mutagenic event in this gene. The mutational levels reported in this study are quite low in comparison to the other studies cited above, and the data reported here. However, the criterion of mutation was the loss of ampicillin resistance. In this case, the appearance of a point mutation generated by $O^6$-methylguanine misincorporation may or may not be deleterious to the function of the beta-lactamase enzyme. Thus, not every base change generated may express itself as a loss of enzyme function. However, the mutants which were characterized all yielded A-T to G-C transition mutations which occurred by the formation of $O^6$-methylguanine-thymine intermediates. These intermediates were repaired by bacterial alkyltransferase to yield G-T mispairs which resulted in the generation of a point mutation during DNA replication. It is important to note that in this study all of the mutation frequencies observed were in isolates obtained from MutH mutants, which are mismatch repair mutants. MutH competent cells did not produce mutation frequencies significantly higher than background. The
transfection of O\(^6\)-methylguanine containing R208 into ada deficient E. coli strains resulted in increased mutagenesis. This mutation efficiency was increased when mutagenized plasmid was treated with dam methylase. The effect of dam methylase treatment overcomes the mismatch repair system of E. coli, which demonstrates that O\(^6\)-methylguanine mutagenesis is influenced by both alkyltransferase and mismatch repair.

In contrast, an oligonucleotide which contained O\(^6\)-methylguanine in the PstI site of M13mp8 generated mutants in the beta-galactosidase gene of bacteriophage M13mp8 (Loechler, Greene, and Essigman, 1984). In these cells, the mutation frequency increased when the host bacterium, E. coli strain HB101, was treated with MNNG. In effect, MNNG depleted the bacteria of alkyltransferase, thus alkylation repair could not take place. The effects of mismatch repair were not significant in this system. It appears the effects of O\(^6\)-methylguanine are dependent upon the alkyltransferase and mismatch repair systems of the host bacterium.

O\(^4\)-Ethylthymine gap directed mutagenesis in this study is enhanced by the effects of alkyltransferase but not effected by the mismatch repair system to the extent described by Topal. This is due to the fact that the amount of alkyl base insertion is at 3 positions, maximum, and that the process of replication occurs within the first two minutes after transfection. Therefore, the miniscule amount of T-G mismatch and the element of time in transformation helps the O\(^4\)-ethylthymine containing plasmids escape mismatch repair.
Various methods of oligonucleotide directed mutagenesis have also yielded high mutational frequencies in the sites being altered. The fundamental difference between oligonucleotide directed mutagenesis and gap misrepair mutagenesis is that oligonucleotide directed mutagenesis uses oligonucleotides which already contain the base anomaly in question, whereas gap misrepair depends extensively on the ability of a DNA polymerase to efficiently misincorporate a mutagenic base at a position.

**CONCLUSIONS**

The efficiency of $\text{O}^4\text{-ethylthymidine}$ gap misrepair mutagenesis is dependent on the ability of the DNA polymerase to misincorporate the alkylated base opposite guanine residues. The incorporation of $\text{O}^4\text{-ethylthymine}$ across from adenine results in low mutational events because of alkyltransferase repair. Thus, the potential for nucleotide pool alkylation *in vivo* to generate mutational misincorporations is a very real consideration in the scope of mutations induced by alkylating mutagens. Also, the ability of the *E. coli* alkyltransferase to repair $\text{O}^4\text{-alkylthymines}$, especially the ethyl derivative, is substantially lower than that for $\text{O}^6\text{-methylguanine}$. Thus, in this system using gap misrepair mutagenesis, $\text{O}^4\text{-ethylthymidine}$ is a potent mutagenic lesion capable of inducing a high level of mutagenic events.

The mutagenic efficiency of $\text{O}^4\text{-ethylthymine}$ misincorporation is influenced by several factors. The fidelity of DNA polymerase activity during synthesis has a profound effect on the mutagenic
frequencies seen. The error prone polymerase, AMV DNA polymerase, has the highest mutation efficiency. The weakly proofreading polymerase from *M. luteus* has a lower mutation frequency. *E. coli* DNA polymerase Large Fragment has the lowest value which is a reflection of the efficient proofreading activity of this enzyme. The effect of alkyltransferase is beneficial when the repair of the alkylation yields a normal T-A base pair. However, alkyltransferase only serves to fix a guanine-\(\text{O}^4\)-ethylthymine pair into a G-T mispair. Therefore, alkyltransferase does not decrease the mutagenic effects of \(\text{O}^4\)-ethylthymine-guanine misincorporations, and may enhance them. The 3′-5′ exonuclease activity of T4 DNA polymerase has also been shown in this study to be an effective mutational mechanism. This process allows \(\text{O}^4\)-EtTTP to be misincorporated into DNA by removing normal pyrimidine bases and creating an opening for \(\text{O}^4\)-EtTTP to be inserted. Finally, the effects of polymerase fidelity and 3′-5′ exonuclease activity are influenced by pyrimidine deoxynucleotide triphosphate pool concentrations. In all of these cases, \(\text{O}^4\)-EtTTP has been shown to substitute for the normal pyrimidine bases, and also competes with them for incorporation.

Gap misrepair mediated incorporation of \(\text{O}^4\)-ethylthymine into DNA is a potent mutagenic event which generates transition mutations which can be demonstrated by the generation of restriction endonuclease resistant transformants. This is a simple, efficient, and highly sensitive system for determining the mutagenic effects of \(\text{O}^4\)-ethylthymine *in vivo*. 
BIBLIOGRAPHY


Jensen and Reed, 1978***


Lindahl, T. and Nyberg, B. (1972) Rate of depurination of native DNA. Biochemistry 11, 3610.


