SYNTHESIS AND CHARACTERIZATION OF OLIGONUCLEOTIDES CONTAINING DEOXYXANTHOSINE: A PROBE FOR THE MUTAGENIC AND GENOTOXIC ACTIVITY OF AN OXIDIZED DNA BASE

by

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(1991)

SUBMITTED TO THE DIVISION OF TOXICOLOGY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF SCIENCE
IN TOXICOLOGY

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

SEPTEMBER 1995

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Division of Toxicology
SEPTEMBER 15, 1995

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ACKNOWLEDGEMENTS

I have progressed far from the hardships of my youth. Every educational degree I earned involves numerous caring people for whom I shall always praise with high esteem. At times while at MIT, the decision to leave California for Massachusetts seemed ludicrous. Departing familiar surroundings with a supportive family base and community for the opportunity to attend a world-class research institution was a difficult decision. The years at MIT have been challenging and at times painfully gruelling. The daily discovery of weaknesses and strengths has shaped a new attribute of my nature. My friends from the Essigmann lab group have been helpful and supportive during my stay at MIT. I particularly extend my gratitude to: Professor John Essigmann who patiently advised and encouraged the completion of this thesis, Mr. William Kobertz who synthesized the xanthine-containing oligonucleotide necessary for the project, Ms. Elisabeth Bailey, Dr. Marjie Solomon and Ms. Elizabeth Trimmer who gave essential encouragement, research guidance and patience leading to an enhancement of my educational experience. Most importantly, my wife Lucy deserves more credit than I could describe for enduring my despair and uplifting my determination to surpass my shortcomings. She will always be my best friend.

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ABSTRACT

It has been shown that nitric oxide ($NO^{\cdot}$) directly exposed to nucleic acids causes deoxypurine deamination leading to the formation of deoxyinosine and deoxyxanthosine *in vitro*. This dissertation describes the synthesis and characterization of oligonucleotides containing two deaminated purine products, deoxyxanthosine (dX) and deoxyinosine (dI). A methodology to assess the mutagenic potential of these nucleosides *in vivo* by using site-specific mutagenesis technology is proposed.

To address quantitatively the issue of deaminated purines leading to G→A or A→G mutations, xanthine and inosine were chemically synthesized and placed into a fragment of the *lacZ* gene of the M13 bacteriophage genome. These modified oligonucleotides containing dX or dI were characterized by using high pressure liquid chromatography, enzymatic digestion and mass spectrometry. Mutations induced by these modified oligonucleotides will give rise to *amber* codons providing a convenient means of color selection in an *amber* suppressor host. Future research using this methodology will provide a quantitative investigation of mutations induced by xanthine and inosine in *Escherichia coli*.

Thesis Supervisor: Dr. John M. Essigmann

Title: Professor of Chemistry and Toxicology

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LIST OF ABBREVIATIONS

Ap	apurinic/apyrimidinic
bp	base pair
BSA	bovine serum albumin
CIP	calf intestinal phosphatase
dA or A	deoxyadenosine
DBU	1,8 diazabicyclo[5.4.0.]undec-7-ene
dC or C	deoxycytidine
dI or I	deoxyinosine
dG or G	deoxyguanosine
ds	double stranded
dT or T	deoxythymidine
dX or X	deoxyxanthosine
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	disodium salt of ethylenediaminetetra-acetic acid
ES-MS	electron-spray mass spectrometry
EtBr	ethidium bromide
form I	supercoiled DNA
form I0	covalently closed circular ds DNA
form II	nicked circular ds DNA
form III	linear ds DNA
IPTG	isopropylthio-β-D-galactoside
HPLC	high pressure liquid chromatography
LB	Luria broth
LD-MS	laser deabsorption mass-spectrometry
M13mp19+1	bacteriophage genome with a refractory PstI site
M13mp19+6	bacteriophage genome with a new PstI site
M-NO	metal bound nitric oxide
NO·	nitric oxide radical
NOS	nitric oxide synthetase
NPE	p-nitrophenyl ethyl
PEG	polyethylene glycol, average MW = 8000
phage	M13 bacteriophage
rf	replicative form
RS-NO	nitric oxide conjugated thiol group
S. cerevisiae	*Saccharomyces cerevisiae*
ss	single stranded
SVPD	snake venom phosphodiesterase
TAE	200 mM Tris, O.57 glacial acid, O.2 mM Na_2EDTA
TBE	89 mM Tris, 89 mM boric acid, 0.2 mM Na_2EDTA

TE	10 mM Tris-HCl (pH 8.0), 1 mM EDTA
Tris-HCl	tris(hydroxymethyl)aminomethane-hydrochloride
UV	ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
YT	yeast-tryptone media

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I. INTRODUCTION

The biological and chemical properties of nitric oxide ($NO\cdot$) have been the subject of intense research. New findings have identified nitric oxide as an important constituent of neuron transmission, immune defense and many other physiological functions in man. As result of these findings, $NO\cdot$ was named "molecule of the year" by the journal *Science* in 1992. In moderate concentrations nitric oxide serves as a secondary messenger or an immunological cytotoxin. However under conditions of prolonged exposure to nitric oxide, such as chronic inflammation, DNA damage and mutations could arise. The possibility of these events has led to further studies as to the deleterious effects of this short-lived reactive molecule. Nitric oxide exposed to naked DNA leads to the formation of two deaminated purines: deoxyxanthosine (dX) and deoxyinosine (dI) (Nguyen 1992). Other researchers have directly exposed $NO\cdot$ to organs, cells, polynucleotides and DNA bases. Their findings support the conclusion that nitric oxide combined with oxygen leads to a deamination reaction on DNA bases. Unfortunately direct exposure of nitric oxide to nucleic acids produces numerous oxidative DNA lesions, making the specific contribution of each mutation from deaminated deoxypurine lesions impossible to measure. To address this issue, site-specific placement of a chemically synthesized oligonucleotide containing dX or dI would allow for unambiguous characterization of mutations, mutation frequency and lesion repair from these two deaminated purines. Nucleic acid mutations could be isolated and quantified using the well characterized *lacZ* gene mutation assay. The *lacZ* gene of the bacteriophage M13 could carry the modified oligonucleotide containing dX or dI and infect

Escherichia coli hosts. The analyzed *in vivo* mutations would improve existing knowledge of the possible genotoxic effects of nitric oxide. Oxidative DNA lesions and repair, nitric oxide attribution and the M13 bacteriophage system are reviewed. The chemical synthesis of modified purine oligonucleotides, genome design, construction and characterization are described in detail.

II. LITERATURE SURVEY

A. Oxidative stress: Daily exposure and reactive oxidants

Oxidative stress can give rise to the formation of modified DNA bases, sugars and DNA strand breaks (Hutchinson, 1985). In general, nucleic acid modifications can be formed by direct ionization of DNA, or by the indirect effect caused by reactive species, in most cases free radicals, which are produced in the vicinity of the DNA. The indirect class of DNA damage results from exposure to both endogenous and exogenous oxidizing agents. Reactive oxygen species, in the forms of superoxide ion ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide(H_2O_2) and nitric oxide ($NO^{\cdot}$) metabolites are formed *in vivo* as a byproduct of normal aerobic metabolism (see reviews Joenje, 1989; Sies, 1991; Moncada et al., 1991). Oxidizing species are also formed during radiation exposure and through xenobiotic metabolism of organic carcinogens. A common and reactive oxygen species generated by water homolysis or metal-catalyzed decomposition of hydrogen peroxide is the hydroxyl radical (Halliwell and Gutteridge, 1989). The superoxide anion radical and conjugated acid forms are ubiquitous in living systems but have low reactivity toward nucleic acid in a hydrophilic environment (Cadet and Teoule, 1978). Similarly, hydrogen peroxide arising from the dismutation of $O_2^{\cdot-}$ has the ability to diffuse passively through cell membranes but is only weakly reactive with nucleic acids (Cadet, 1994). Reactions involving DNA base deamination are believed to occur spontaneously or through reactive nitric oxide species.

In general lesions formed in DNA by oxidants are very similar to those formed by ionizing radiation. As a process of evolution, organisms have developed numerous and complex defenses against oxidative and radiation damage. Nevertheless, it has been estimated that the genome of a rodent and human cell receives about 10^5 and 10^4 oxidative hits per day respectively (Park et al., 1992; Ames et al., 1993). Oxidative DNA damage leading to mutations has been implicated in a variety of degenerative disorders including cancer and aging (Loeb, 1989; Shigenaga et al., 1994). Below to introduce the topic of oxidative stress research, DNA lesions induced by hydroxyl radicals or DNA base deamination are focused upon. In addition, methodologies for measurement of these oxidative lesions are mentioned with examples of DNA base excision repair of two commonly found oxidative lesions.

Methodologies

HPLC or gas chromatography with appropriate spectroscopic detection techniques have allowed for detection and characterization of numerous DNA base and sugar lesions (see Figure 1). The two following methods have been used to measure oxidative lesions *in vitro* by exposing isolated DNA, cells or organs to specific oxidizing agents. Gas chromatography-mass spectrometry (GC-MS) has successfully monitored the formation of damage at several purine and pyrimidine residues of DNA and chromatin exposed to radical oxidizing agents (Dizdaroglu and Gajewski, 1990; Halliwell and Dizdaroglu, 1992). Unfortunately a problem with the GC-MS approach is the high background levels of oxidized lesions produced subsequent to acid hydrolysis, derivation and enzymatic digestion of the oxidized DNA base. Another useful approach is the HPLC-electrochemical detection

method, which yield 3-8 times lower background levels than the GC-MS approach (Floyd et al., 1986; Wagner et al., 1992). Both techniques are used for the measurement of oxidative DNA damage within cells generally requiring an assay detection threshold close to one lesion per 10^5-10^6 normal bases. Alternatively, assessment of oxidative DNA damage in humans and animals has been achieved by the global measurement of oxidized bases and nucleosides released in urine as a result of DNA repair (Ames and Gold, 1991; Simic and Bergtold, 1991).

Mutation assays

The assessment of *in vivo* DNA mutations induced by specific oxidants can be accomplished by number of methods. One method involves global exposure of the suspected mutagen to whole cells or organisms. Two examples are the *Salmonella*/mammalian microsome test which is based on reversion of auxotrophs (Maron and Ames, 1983) and the hypoxanthine-guanine phosphoribosyltransferase (HPRT) mutants selected by resistance to purine analogues in Chinese hamster or human cells (DeMarini et al., 1989). In addition, various bacterial and bacteriophage gene mutation assays have been developed in which the nucleic acids are directly exposed to a specific oxidant and the affected DNA is re-introduced into a host system. One standard system is the M13 bacteriophage *LacZ* gene assay, which has been used to measure the mutagenic specificity of oxygen radicals produced by human leukemia cells in culture (Reid and Loeb, 1992). A chemically synthetic approach is site-specific mutagenesis, which incorporates a single oxidative product into a gene *in vitro* and measures the mutagenic contribution of the individual lesion *in vivo* (Basu and Essigmann, 1988).

Deoxypyrimidines

The most susceptible of the DNA bases to free radical mediated oxidative damage *in vitro* is thymine. The 5,6 double-bond of thymine is subject to radical attack with a slight preference for the C-5 position (Fujita and Steenken, 1981). Unstable thymine hydroperoxides are initially formed but could become further reduced to 5,6-dihydro-5,6-dihydroxy thymine, otherwise known as thymine glycol. Thymine glycol has been found to be the major radiolysis product when thymine or DNA were irradiated *in vitro* (Teoule and Cadet, 1978) and has been identified in human and animal urine (Adelman et al., 1988). *cis*-Thymine glycol was site-specifically positioned in a single-stranded genome of the M13 bacteriophage and found to form a T→C transition (Basu et al., 1989). Other $OH^{\cdot}$ induced products are frequently *cis* and *trans* isomers of 6-hydroperoxy-5-hydroxy-5,6 dihydrothymine and 5-hydroperoxy-6-hydroxy-5,6-dihydrothymine. The $OH^{\cdot}$ also attacks the 5,6 double bond of cytosine forming an unstable cytosine and 2'-deoxycytidine hydroperoxides. Further examination of one of the oxidized cytosine (5'hydroxy-6-hydro-2'-deoxycytidine) revealed a disposition of C→T transitions (Wagner, 1992). Other known oxidized pyrimidine products are: 5-hydroxyhydantoin, 5-hydroxy-6-hydrothymine, 5,6-dihydrothymine, 5-hydroxy-5-methylhydantoin, and cytosine glycol.

Deoxypurines

In general, purines are thought to be less affected by hydroxyl radical exposure than pyrimidines. However oxidized purines are formed and usually occur as:

8-oxo-7,8-dihydro-2'-deoxyguanine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (fapy guanine), 4,6 diamino-5-formamidopyrimidine (fapy adenine) and 7,8-dihydro-8-oxoadenine. For $OH^{\cdot}$ damaged adenine, the 7,8-dihydro-8-oxoadenine is the most abundant lesion formed and has been isolated in human urine (Stillwell et al., 1989). Spectrometric studies have shown the 7,8-dihydro-8-oxo-2'deoxyadenosine to exist mainly as a keto tautomer with the proton on the N7 atom (Cho and Evans, 1991; Guschlbauer et al., 1991). Site-specific evaluation of 7,8-dihydro-8-oxoadenine revealed that it could mispair with dG implying an A→C transversion (Shibutani et al., 1993) but it is not mutagenic *in vivo* (Wood et al., 1992). The main products of $OH^{\cdot}$ mediated decomposition of deoxyguanosine are 2,2-diamino-4-[(2-deoxy-β-D-erthyo-pentofuranosyl)amino]-5-(2H)-oxazolone and 2-amino-5-[2-deoxy-β-D-erthyopento-furanosylamino]-4H-imidazole-4-one (Cadet et al., 1991). The 7,8-dihydro-8-oxo-2'-deoxyguanine lesion has been found as a urinary excretion product in man (Degan et al., 1991). NMR studies have demonstrated that 7,8-dihydro-8-oxo-2-deoxyguanosine exists in solution as the 6,8-diketotautomer with a preferential *syn*-glycosidic conformation (Culp et al., 1989; Cho et al., 1990). Site-specific incorporation of 7,8 dihydro-8-oxo-2'deoxyguanosine has been accomplished and it has been shown that the lesion induces a G→T transversion *in vivo* (Wood et al., 1990).

DNA deamination

DNA model studies have shown that cytosine components are much more susceptible to hydrolytic deamination than purine constituents under neutral and slightly acidic conditions (Shapiro and Klein, 1966). Cytosine deamination is known to

form uracil. Under physiological conditions the cytosine deamination rate constant for single and double-stranded DNA has been measured to be 1 X 10^{-10} and 7 X 10^{-13} per second (Frederico et al., 1990). Curiously cytosine deamination is believed to be 3-5 times slower than 5-methylcytosine conversion to thymine (Ehrlich et al., 1990). Spontaneous base deamination has been shown to occur at 5-methylcytosine residues in the *lacI* gene of *E. coli*, giving rise to base substitution "hot spots" (Coluondre et al., 1978) believed to occur preferentially at GC sequences. 5-Hydroxy-methyluracil bases has been found in human urine (Faure et al., 1993). Other oxidative lesions of uracil derivatives have been identified such as: 5,6-dihydroxy-5,6-dihydro-uracil (uracil glycol), 5-hydroxy-2'-deoxyuridine, 5-hydroxyl-methyluracil and 5-formyluracil. The *cis* and *trans* configurations of 5,6-dihydroxy-5,6-dihydrouracil have been shown to arise from the deamination of cytosine glycol (Polverelli and Teoule, 1974). The purine base in DNA is more resistant to spontaneous hydrolysis than pyrimidine constituents. However purine constituents were found to be more easily deaminated by nitrous acid than pyrimidine analogues (Shapiro and Yamaguchi, 1972). The environmental pollutant and bioregulator nitric oxide is mutagenic and has been shown to deaminate purine residues (Wink et al., 1991; Arroyo et al., 1992). Nitric oxide exposure to naked DNA yields two deaminated purine products, namely deoxyinosine and deoxyxanthosine (Nguyen et al., 1992). A more comprehensive review of nitric oxide chemistry and biology are presented in later sections.

DNA base excision repair

In general, mutations could arise from the misincorporation

of DNA bases by DNA polymerase or by misrepair or no repair of DNA lesions. One way cells remove oxidative lesions is by base excision repair. Glycosylases cleave the bond between deoxyribose and a damaged or inappropriate base leading to the formation of an apurinic or apyrimidinic (AP) site. Specific DNA glycosylases remove particular lesions. A closer look at the incision-dependent repair reveals four steps: the phosphodiester-bond cleavage (incision), removal of one or more nucleotides including the damaged bases (excision), replacement of the nucleotides by DNA polymerase (resynthesis) and formation of a phosphodiester bond linking the newly synthesized region or "patch" to the rest of the DNA strand (ligation). Two well known DNA base excision systems in *E. coli* are examined further with respect to thymine glycol and 8-oxoguanine repair.

Thymine glycol excision repair

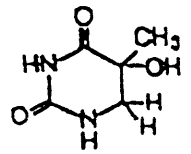
In vitro studies of thymine glycol adducts has shown that this lesion blocks DNA replication (Rouet and Essigmann, 1982). Such an event obviously could lead to cell death. To cope with the dangers of thymine glycol accumulation, bacterial cells produce at least two specific base excision proteins. The first repair protein is endonuclease III encoded by the *nth* gene. This enzyme is associated with a DNA glycosylase specific for 5,6-hydrated thymine moieties, which include thymine glycol and uracil moieties (Demple and Linn, 1980; Kather and Wallace, 1983). The function of endonuclease III is to hydrolyze the 3'-phosphate and 3'-phosphoglycolate into a 3'-hydroxyl. The *xth* gene also encodes the endonuclease III protein and is sometimes

referred as endonuclease VI. After base hydrolysis, other proteins replace the AP site with the appropriate base as explained earlier. In general endonuclease III is a major AP endonuclease of *E. coli*. Duplication of essential genes is a common feature in bacteria as well as in higher developed organisms.

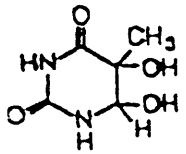
8-Oxoguanine excision repair

The 7,8-dihydro-8-oxoguanine (8-oxoG) adduct can result in a misincorporation of an adenine (A) opposite the 8-oxoG. The *MutY* gene codes for a glycosylase specific for the A:8-oxoG mispair. The function of mutY glycosylase is to remove the misincorporated dA from the A:8-oxoG mispairs that result from error-prone replication past the 8-oxoG lesion (Michaels et al., 1990, 1991). A complement to the MutY protein is the MutM enzyme, also known as the fpg protein. The fpg enzyme is an 8-oxoG endonuclease (Tchou et al., 1991). The repair scenario may follow this pattern: the fpg excises 8-oxoG during the first round of replication. Any remaining lesions could encounter MutY, adenine glycosylase, provided adenine is positioned opposite 8-oxoG. Both the MutY and MutM systems seem to work in concert to increase the opportunity to remove 8-oxoG and prevent GC→TA and AT→CG mutations. Studies using double mutants of *MutM* and *MutY* genes have lead a several fold higher mutation rate than cells missing either glycosylase alone. In addition to base excision other mechanisms exist for cells to survive unrepaired lesions such as: postreplication repair, daughter-strand gap repair, bypass synthesis or trans-lesion synthesis.

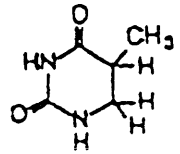
Figure 1. A partial list of pyrimidine and purine products of oxidative damage. Three of the lesions listed (8-hydroxyguanine, 8-hydroxyadenine and thymine glycol) have been characterized as to their mutagenic potential by site-specific mutagenic techniques.



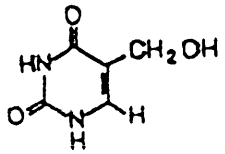
5-hydroxy-6-hydro-thymine



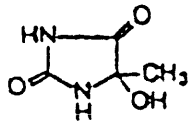
thymine glycol



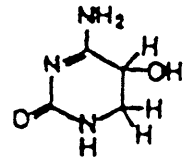
5,6-dihydrothymine



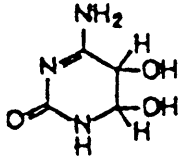
5-(hydroxymethyl)-uracil



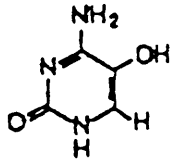
5-hydroxy-5-methyl-hydantoin



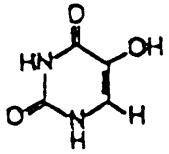
5-hydroxy-6-hydro-cytosine



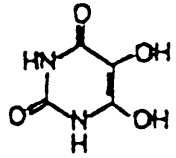
cytosine glycol



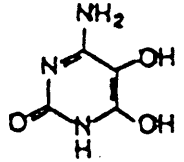
5-hydroxy-cytosine



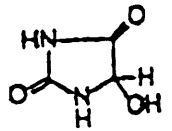
5-hydroxy-uracil



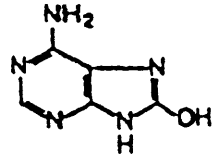
5,6-dihydroxy-uracil



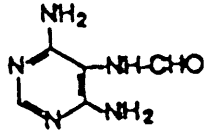
5,6-dihydroxy-cytosine



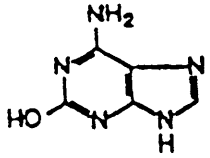
5-hydroxyhydantoin



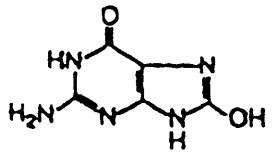
8-hydroxyadenine



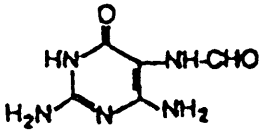
4,6-diamino-5-formamido-pyrimidine



2-hydroxyadenine



8-hydroxyguanine



2,6-diamino-4-hydroxy-5-formamidopyrimidine

B. Biological significance of Nitric Oxide

Mammalian cells as diverse as macrophages (Marletta et al., 1988), neutrophils (Wright et al., 1989), neurons (Bredt and Snyder, 1992) and endothelial cells (Moncada et al., 1988) synthesize nitric oxide from L-arginine while several types of abdominal bacteria produce nitric oxide by reduction of nitrite (Calmels et al., 1985). The function of this compound at low levels is generally beneficial. Nitric oxide in three different functional capacities is reviewed. The subjects are endothelium-dependent relaxation (Furchgott and Zawadzki 1980), neurotransmission (Garthwaite et al., 1988), and cell-mediated immune response (Nathan and Hibbs, 1991). Schematic representation of NO· activity is shown in Figure 2.

Nitric Oxide Synthase

Nitric oxide is produced endogenously by the action of nitric oxide synthase (NOS). The production of nitric oxide can be constitutive or inducible dependent on cell function (see review Moncada et al., 1991). Brain cells and endothelial cells have the constitutive form activated by the regulatory protein calmodulin (Busse and Mulsch, 1990), which itself is activated by an influx of calcium ions into the cell. NO· released under physiological conditions by NOS require an electron donor (NADPH) and the cofactor tetrahydrobiopterin, which are used for the reaction of molecular oxygen and the amino acid L-arginine. The interaction involves removal of the nitrogen atom from arginine and the reaction with an oxygen atom to form NO· and citrulline (reviewed Marletta, 1994). The availability of L-arginine is well regulated as demonstrated by

endothelial cells deprived of L-arginine yet are still able to synthesize this amino acid from endogenous sources (Hecker et al., 1990; Sessa et al., 1990). Researchers have extensively used arginine analogues such as N-monomethylarginine (NMMA) as inhibitors of NOS to help elucidate the enzyme action.

Endothelial Derived Releasing Factor

Nitric oxide secreted by endothelial cells is generally referred to as endothelium-derived relaxing factor (EDRF). EDRF and $NO^{\cdot}$ have been found to be the same molecule (Ignarro and Furchgott 1988). EDRF functions as an antagonist to smooth muscle contraction by inhibiting receptor agonist-evoked Ca^{2+} influx from intracellular stores (Olsen et al., 1976; Knowles et al., 1989). The believed mechanism is dependent on EDRF binding to the heme component of the enzyme guanylate cyclase (Miki et al., 1977). The activated enzyme then synthesizes the molecule cyclic guanosine monophosphate (cGMP). The cGMP acts as a signal that ultimately induces muscle relaxation by cGMP-dependent protein kinase inhibition of receptors that gate intracellular Ca^{2+} concentrations. In the cardiovascular system the release of $NO^{\cdot}$ acts as a general adaptive mechanism whereby the vascular endothelium responds to changes in its environment and regulates blood flow and blood pressure (Radomski et al., 1990). The presence of EDRF induces vascular smooth muscle relaxation and consequently lowers blood pressure. EDRF also inhibits platelet aggregation and adhesion (Azuma et al., 1986). Some researchers believe $NO^{\cdot}$ is bundled to a carrier molecule that prevents its reaction with oxygen (Stamler et al., 1992). EDRF interactions in aerobic systems are discussed in the $NO^{\cdot}$ chemistry section.

Neurotransmission

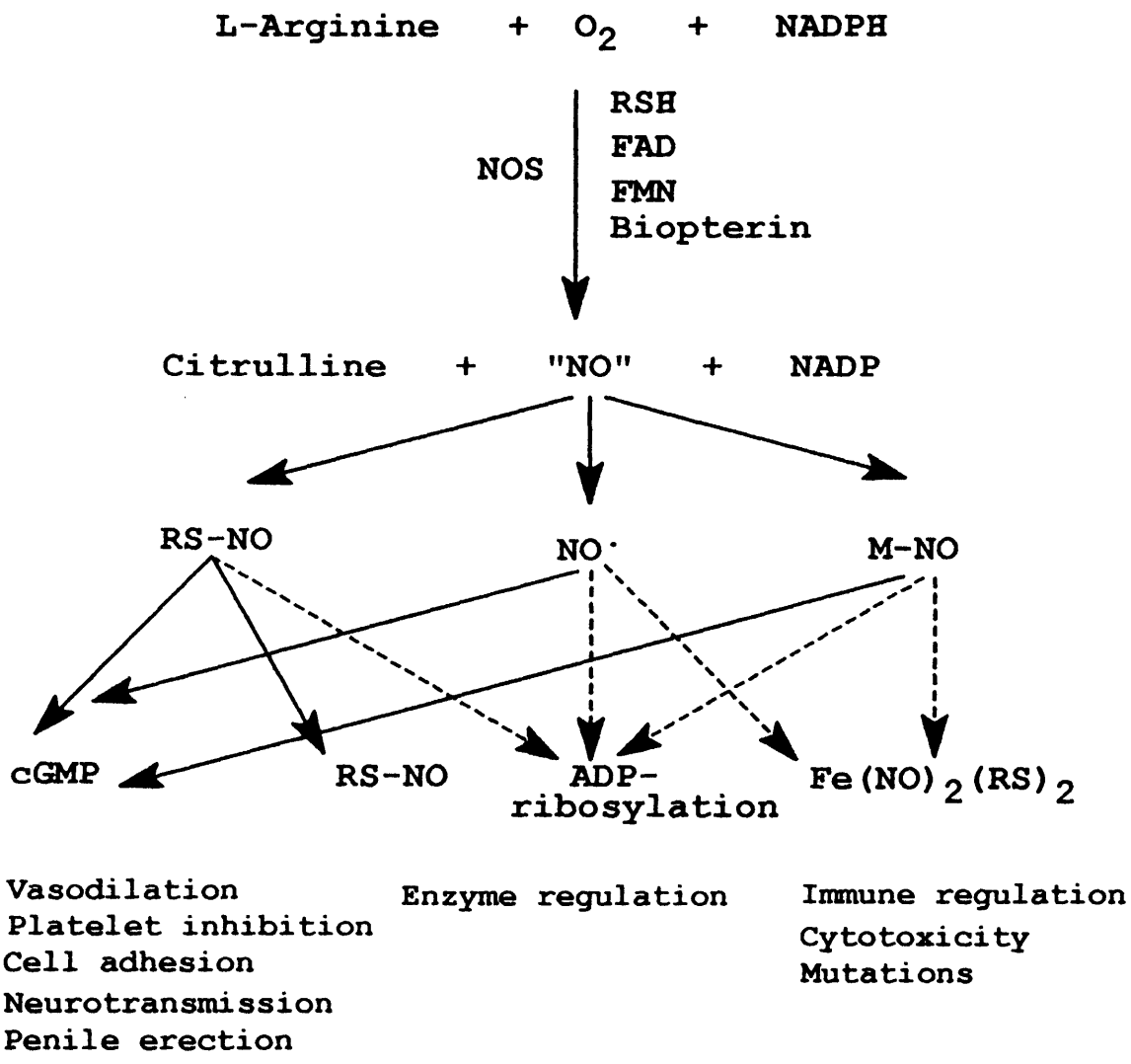
The importance of the L-arginine:NO pathway in the nervous system has yet to be fully established. $NO^{\cdot}$ in neurons is not stored, but rather synthesized on demand; its general action is mediated by diffusion to intracellular targets rather than by binding to specific plasma membrane receptors and there is no known inactivation or uptake mechanism (Schmidt and Walter, 1994). The function of synaptic plasticity has been suggested by $NO^{\cdot}$ interaction with the N-methyl-D-aspartate (NMDA)-type glutamate receptor which could attenuate long term potentials (Schuman and Madison, 1991). In the long-term potentiation scenario, glutamate is released from a presynaptic neuron and binds to the NMDA receptor on the postsynaptic neuron. The receptor binding releases a retrograde messenger ($NO^{\cdot}$) that diffuses back to the presynaptic neuron, causing a subsequent increase in the level of cGMP molecule and promotes the release of more glutamate from the presynaptic terminal. The cycle repeats itself and enhances the synaptic connection. The human body coordinates $NO^{\cdot}$ expression to cause physiological change. Neurons use $NO^{\cdot}$ to regulate transmitter release of adjacent neurons (Meffert el al., 1994) and EDRF affects cerebral blood flow to match neuronal activity all in conjunction with bronchial epithelial and endothelial cells for appropriate ventilation and perfusion (Gaston et al., 1994).

Immunology

Resistance to infection and cancer can be enhanced in a non-specific way by bacterial products, a fact known for more than a century (Coley, 1893). A later study revealed macrophages are able to give rise to the observed resistance

(Old et al., 1961). Macrophages (killer cells) recognize and destroy host cells that have become infected with a pathogen. Killer cells respond by ingesting the infected cell and release cytokines that further activate other cells or release toxic substances that directly kill the infected cell. $NO^{\cdot}$ has been suspected to be related to this cytotoxicity (Hibbs et al., 1990). Macrophages possess the inducible type of NOS. The $NO^{\cdot}$ expressed by the cell-mediate immune response is always non-specific and calcium independent (Nathan and Xie, 1994). The inducible NOS produces much greater amounts of nitric oxide than the constitutive form. This factor is key to the differing roles of $NO^{\cdot}$ in the body. Stimulated macrophages secrete nitric oxide to a level of 1000 times above normal constitutive NOS expression (McCall et al., 1989). Nitric oxide is also related to the cytotoxicity of tumor cells (Nathan and Hibbs, 1991), which supports the immune-surveillance theory of cancer. The theory states that an uncompromised immune system kills tumor cells before they become malignant. Presented here are the beneficial attributes of nitric oxide. However, $NO^{\cdot}$ also has detrimental characteristics as presented in the toxicology section.

Figure 2. The biological role of nitric oxide in humans. This radical serves the body as a secondary messenger with vastly different functions according to tissue type and amounts secreted. Nitric oxide secretion is both inducible and constitutively produced. Typically $NO^{\cdot}$ serves a normal function in cell signaling, enzyme regulation and immune interactions. However $NO^{\cdot}$ could also induce mutations in neighboring nucleic acids under conditions in which macrophages and/or neutrophils secrete excessive amounts of nitric oxide. This occurs in the case of chronic inflammation. The following symbols NOS, RS-NO, M-NO denote nitric oxide synthetase, nitric oxide bound to a thiol group and the metal bound nitric oxide species, respectively as noted by Stamler et al., 1992.


L-Arginine + O_2 + NADPH
NOS
RSH
FAD
FMN
Biopterin
Citrulline + "NO" + NADP
RS-NO
NO·
M-NO
cGMP
RS-NO
ADP-ribosylation
$Fe(NO)_2(RS)_2$
Vasodilation
Platelet inhibition
Cell adhesion
Neurotransmission
Penile erection
Enzyme regulation
Immune regulation
Cytotoxicity
Mutations

C. Nitric Oxide chemistry

Nitric oxide is an inorganic gas ubiquitous in the human body. Under physiological conditions NO· can cause DNA damage in several ways: 1) interactions with electrophiles arising from metabolism of N-nitroso compounds, 2) nitrosation of amines on nucleic acid bases leading to deamination or 3) attack by active species arising from reactions of nitric oxide with endogenous oxygen radicals (see Figure 3).

Nitric Oxide half-life and diffusion distance

Cells do not appear to possess an enzymatic mechanism for NO· removal (Schmidt and Walter, 1994). One reason could be the short half-life of this molecule. Nitric oxide is only a two atom molecule and has electrical neutrality, which allows its easy passage through biological membranes facilitating its action in cell signaling. The half-life of nitric oxide and the diffusion length seems to be largely dependent on the cellular location, concentration of nitric oxide and the local environment. For example, $t_{1/2}$ has been reported as short as 0.1 sec to 4 sec or longer at physiological conditions (Lancaster, 1994) and diffusion distances range from $\leq$ 160 μm in cell-cell communication to $\geq$500 μm for cell to blood diffusion (Saran and Bors, 1994). Given cell diameters of 10-20 μm/cell, NO· has the capacity to travel to as few as 8 to as much as several thousand cells. Reaction with protein-thiol groups form S-nitroso derivatives that function like NO· and have implications for a longer-lived molecule (Stamler et al., 1992).

N-Nitroso compounds

Endogenous formation of N-nitroso compounds have been extensively studied. N-Nitrosamines are well-known chemical carcinogens that are metabolized to strong alkylating electrophiles that react with DNA at several nucleophilic sites (reviewed Zimmerman, 1977). The alkylation spectra vary from agent to agent, but reactions at the N-7 and O-6 positions of guanine and the N-3 position of adenine typically predominate (Pegg, 1977).

Historically, amine nitrosation in lab settings were accomplished with acidic nitrite. The actual nitrosating species for amines is N_2O_3 (Pegg, 1977; Challis et al., 1989). The overall rates for amine nitrosation reactions go through maxima near pH 3.5, reflecting increasing concentrations of nitrosating species and a decreasing concentration of free amine as the medium become more acidic (Mirvish, 1975). Nitric oxide and the resulting nitrosations are examples of chemical pathways that were elucidated by Challis and co-workers, 1989. An effective nitrosating agent for primary and secondary amines under non-acidic conditions is N_2O_3. Reactions with secondary amines form N-nitrosoamines in aqueous and organic solutions *in vitro* and for an number of nitric-oxide generating cell systems in culture, including bacteria (Calmels et al., 1987) and macrophages (Iyengar et al., 1987). Reaction with aryl diazonium ion of the base undergoing deamination with a nucleophilic site on an adjacent macromolecules could lead to crosslinking with other nucleic acids or with proteins (Kirchner et al 1992).

Oxygen radicals related to Nitric Oxide

DNA damage by oxygen radicals that may be formed from nitric oxide has been difficult to establish in detail. There is increasing evidence that such reactions occur. For example, several nitric oxide-related processes can be attenuated by superoxide dismutase, indicating that the superoxide anion radical is involved (Heinzel et al., 1992; Pou et al., 1992). Superoxide alone is weakly reactive to DNA bases (Cadet and Teoule, 1978). Reaction of superoxide with nitric oxide to form peroxynitrite has been demonstrated (Blough and Zafiriou, 1985; Beckman et al., 1990). Also hydroxyl radicals arising from superoxide via Fenton-or Haber-Weiss-like chemistry has been suggested as the active species (Imlay, 1988). Evidence has also been cited that the crucial process involves generation of hydroxyl radical-like compounds (Koppenol et al., 1992) by decomposition of peroxynitrite formed by the direct reaction of superoxide and nitric oxide (Saran et al 1990). However, arguments for oxidants other than the hydroxyl radical and the peroxynitrate anion as the reactive species in the $NO^{\cdot}/O_2$ reaction have been cited by others (Wink et al., 1991, 1993; Pryor et al., 1994).

Assuming peroxynitrite is formed, a number of possible reactions can occur (reviewed by Plumb et al., 1992). Peroxynitrite, with a pKa of 6.8, is protonated in acidic solution to form peroxynitrous acid that can decay rapidly to predominately nitrite. The mechanism has not been completely elucidated but it appears to involve a combination of isomerization and radical formation-recombination. The reaction in base leads to nitrite as the major product along with molecular oxygen and proceeds

slowly unless metal ions are present. The controversy concerning whether peroxynitrite releases hydroxyl radicals *per se* or gives rise to unstable reactive intermediates, (isomers of peroxynitrite anion, Beckman et al., 1992), does not distract from the biological observations that $NO^{\cdot}$ DNA damage is similar to hydroxyl radical induce damage.

Nitric Oxide induced DNA base deamination

Purines and pyrimidines (and their derivatives) containing an amino group treated with nitrous acid yield unstable diazonium salts. The diazo intermediates are readily decomposed to form deaminated compounds such as inosine, xanthosine and uridine respectively, from adenosine, guanosine and cytidine (Kochetkov and Budovskii 1972). As noted in the biology section, $NO^{\cdot}$ is generated *in vivo* by the reactions of NO_2 with oxygen or by the reaction of other nitrogenous precursors (e.g., arginine) with reactive oxygen species. Through subsequent chemical reactions, the potent electrophile N_2O_3 could be formed, which can deaminate cytosine, 5-methylcytosine, guanine and adenine in DNA (Wink et al., 1991) see Figure 4. In particular the suspected deaminated purine products are thought to be deoxyxanthosine and deoxyinosine (Nguyen et al., 1992). The deaminated bases formed by this reaction pose potential mutagenic risks as noted in the toxicology section.

Nitrosative deamination is a well-known consequence of the reaction of primary amines with acidic nitrite (Obiedzinski et al., 1980). The nitrosating species, N_2O_3 generated from nitrous acid, will occur under non-acidic conditions when N_2O_3 is generated in the presence of primary amines (reviewed Tannenbaum et al., 1994). Nitrosation of primary

amines results in rapid deamination via diazonium ions and diazohydroxides. Deamination by this mechanism is possible in principle with most purines and pyrimidines; an exocyclic amino group is the principle structural requirement.

Deoxyxanthine chemistry

Xanthosine with a pK of 5.7 exists in the diketo tautomeric form in aqueous solution at pH 5 and the 6-keto-2-enolate anion structure at neutral and slightly basic pH (Roy and Miles, 1983). When deoxynucleosides are treated with nitrous acid, an important side reaction is cleavage of the N-glycosidic bond in the deaminated products (Kochetkov and Budovskii, 1972). In particular, deoxyxanthosine has been noted to have high acid lability of its N-glycoside bond (Shapiro and Pohl, 1968). Comparison of deaminated deoxypurines at pH 3.35 and 37 °C demonstrated that 56% of deoxyxanthosine is converted in 2 hours into xanthine, whereas deoxyinosine yields 54% hypoxanthine in 24 hours (Shapiro and Chargaff, 1966). The first order velocity constant for acid hydrolysis of 2'-deoxyxanthosine-5'-phosphate and 2'-deoxyinosine-5'-phosphate is 9.7 X 10^{-5} moles/sec and 3 X 10^{-6} moles/sec, respectively (Kochetkov and Budovskii, 1972). Hydrolysis of the N-glycosidic bond in a nucleoside leaves an abasic site that could be cleaved by an endonuclease or by base catalysis to yield a DNA single-strand break. Alternatively, the abasic site could "pair" with adenine during replication (Loeb and Preston, 1986).

Depurinated DNA base mispairs

As noted earlier, deoxyuracil, deoxyxanthosine (dX) and deoxyinosine (dI) are implicated DNA deamination products

produced by nitric oxide that can form DNA mispairs (see Figure 5). Eritja et al. (1986) used *Drosophila melangaster* DNA polymerase α to measure relative nucleoside incorporation rates opposite deoxyxanthosine. Also in the same study, the melting temperature of duplex oligonucleotides containing X:T, X:G, X:C, and X:A basepairs at neutral pH are reported. Thermodynamic stabilities of the mispairs are in the order of T>G>A = C and deoxynucleotide incorporation at the site opposite to dX shows a preference of T>C>A = G. The nucleoside incorporation did not correspond well with base stability except for the X:T mispair. Stability studies done with deoxyinosine mispairs display the order of stability as I:C> I:A> I:T = I:G (Martin et al., 1985; Aboul-ela and Tinoco Jr. 1985). Hypoxanthine behaves approximately as a guanine analog in nucleic acids (Martin et al., 1985). NMR studies confirmed the formation of a dI(*syn*):dG(*anti*) base pair in a B-DNA helix (Oda et al., 1991). The NMR study and a sequence dependent study of two adjacent deoxyinosines (Case-Green and Southern, 1993) support the idea that hypoxanthine can form hydrogen-bonded base pairs with all four normal DNA bases.

Figure 3. Possible pre-mutagenic events pertaining to nitric oxide chemistry. Oxidative damage of DNA bases is believed to involve the Haber-Weiss reaction. $NO^{\cdot}$, oxygen and a transition metal could produce highly reactive hydroxyl radicals leading to oxidative lesions, strand breaks and cross-links. The question mark (?) denotes the uncertainty as to the ultimate reactive species of nitric oxide and oxygen. Alternatively, nitric oxide could also react with primary or secondary amines yielding N-nitroso compounds or a deamination reaction.

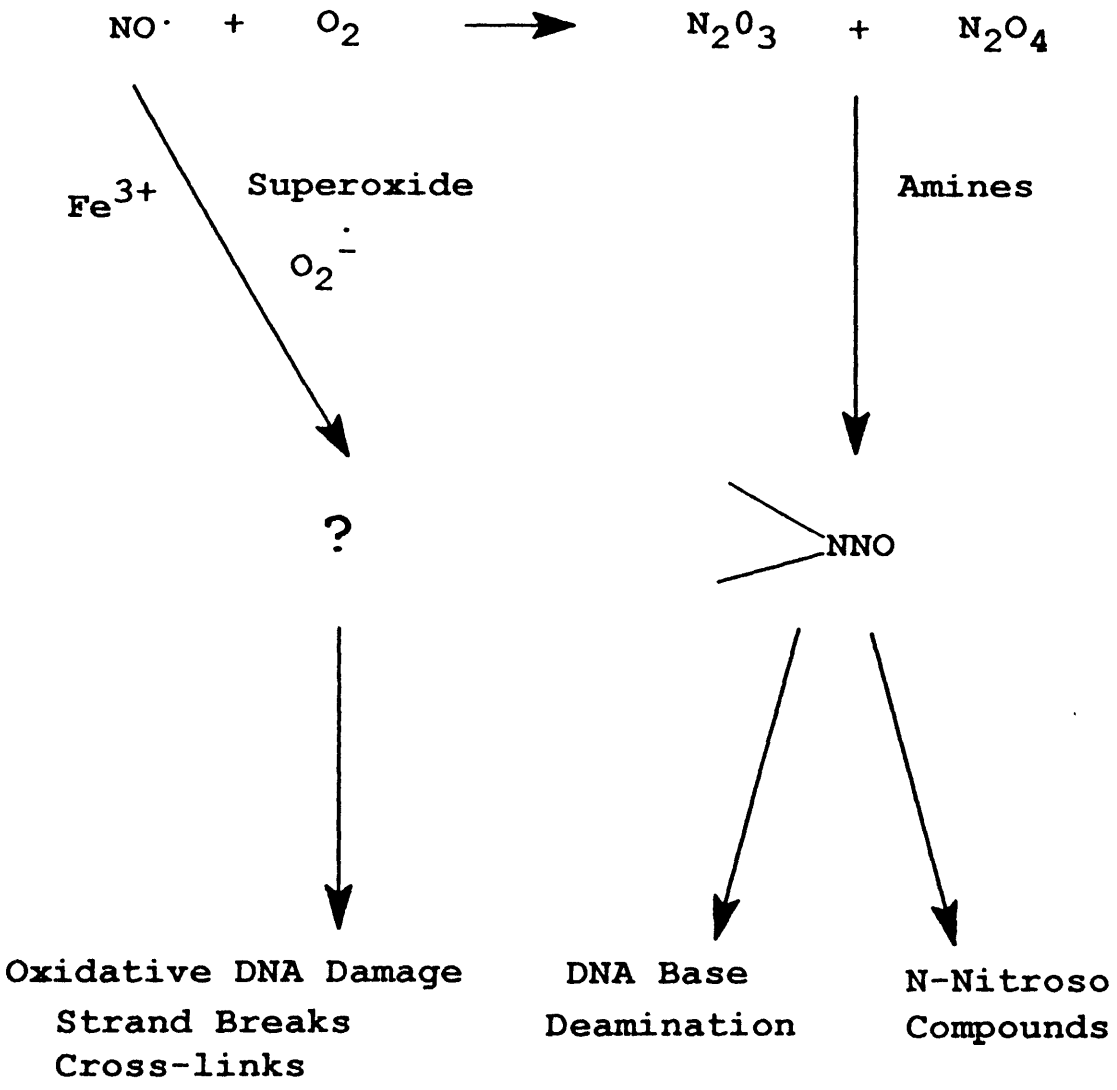
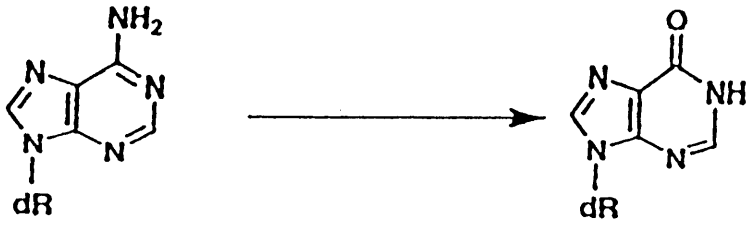

NO· + O2
N2O3 + N2O4
Fe3+
Superoxide
O2·−
Amines
?
NNO
Oxidative DNA Damage
Strand Breaks
Cross-links
DNA Base
Deamination
N-Nitroso
Compounds

Figure 4. Possible DNA base deaminations induced by nitric oxide exposure. Purine deamination is the focus of this study. Specially, the possible mutagenic potential of deoxyxanthosine and deoxyinosine is under study.

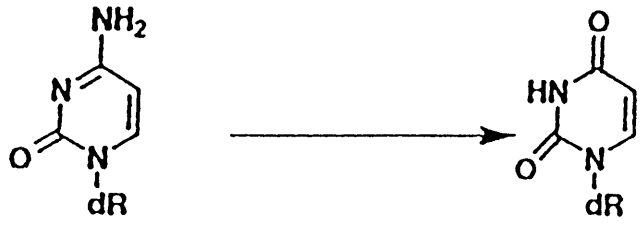
deoxyguanosine

deoxyxanthosine



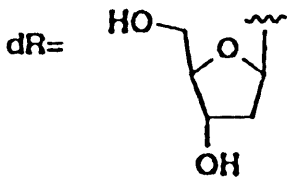
deoxyadenosine

deoxyinosine



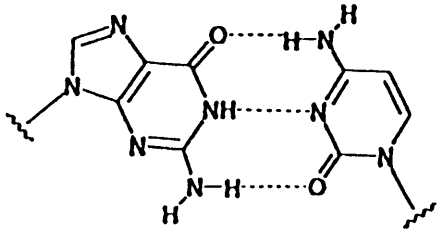
deoxycytosine

deoxyuracil

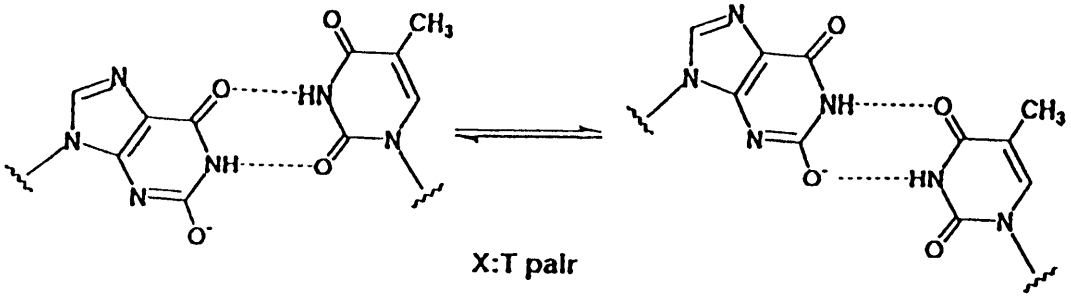


deoxyribose moiety

Figure 5. Wobble base pairing of xanthine and inosine with pyrimidines. Xanthine is believed to base pair with thymine to yield a guanine to adenine transition. Similarly, inosine can mispair with cytosine giving the potential of an adenine to guanine transition.

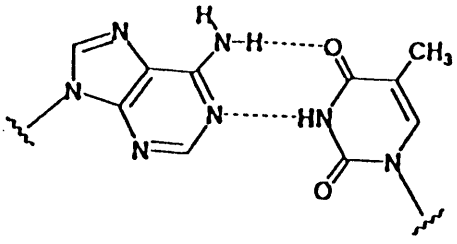


G:C pair

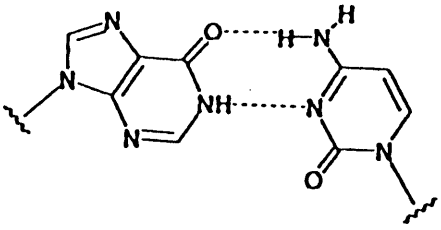


X:T pair

G → A



A:T pair



I:C pair

A → G

D. Nitric Oxide toxicology

Cytotoxicity, cytostasis and DNA damage have been associated with both beneficial and harmful effects of nitric oxide. The ultimate cellular consequence is largely determined by the relative concentration of nitric oxide and cellular sensitivity to this chemical. The two categories of injury are presented along with several suggested examples of possible $NO^{\cdot}$ damage accounting for pathophysiological trauma or disease.

$NO^{\cdot}$ induces cytotoxicity and cytostasis

The immune system deploys $NO^{\cdot}$ to kill or inhibit growth of foreign organisms. Macrophages, which elicit cytotoxic effects, have been measured to secrete $NO^{\cdot}$ on the order of 5×10^3 to 5×10^4 molecules/cell-sec (Marletta et al., 1988). Ironically the generated $NO^{\cdot}$ could also have adverse effects on host cells induced to express the $NO^{\cdot}$ synthase or in adjacent cells. Macrophages in which this pathway has been induced show signs of $NO^{\cdot}$ dependent toxicity (Higgs et al., 1990). The mechanism of $NO^{\cdot}$ leading to cell death or cellular dysfunction is being actively pursued.

One of method to explain the toxicity involves nitric oxide binding to metal atoms (reviewed by Lancaster, 1992). Research by Nathan and Hibbs (1991) demonstrated that cells killed by macrophages lose the function of certain iron-bearing enzymes. This physical property of nitric oxide binding to the metal-center of enzymes could possibly inhibit catalysis involved in cellular respiration. This action suggests that target cells stop working properly and lead to cell starvation. Excessive $NO^{\cdot}$ could also induce

hyper-activity of normal physiological functions. For instance septic shock, an affliction initially caused by bacterial toxins that enter the blood circulation, triggers a systemic immune response that lowers blood pressure (EDRF function) to the critical point of vital organ failure.

In a non-immune response, Bredt and Snyder (1992) proposed a model to account for massive neuron death after certain types of head trauma. Excitotoxic death is a condition where large amounts of the excitatory amino acid glutamate released into synaptic sites causes neurons to excite themselves to death. The release of glutamate activates receptors and a large amount of calcium influx ensues, which activates nitric oxide synthase through the enzyme's dependence on calmodulin. Consequently, the massive amounts of nitric oxide released into the region probably destroy neurons by binding the metalloproteins significant to cellular metabolism.

DNA mutation

Another possible mechanism to account for the etiology of human diseases is genetic mutation (Zimmermann, 1977). Nitric oxide exposed to *Salmonella* has been shown to be mutagenic (Arroyo et al., 1992). As noted in the chemistry of nitrous acid, deamination of DNA residues are prevalent after NO· exposure. AT→GC transitions could arise from adenine conversion to hypoxanthine (Ohtsuka et al., 1985; Hill-Perkins et al., 1986; Kamiya et al., 1992a) and GC→AT transitions could result from deamination of cytosine to uracil (Duncan and Miller, 1980), of 5-methylcytosine to thymine (Coulondre et al., 1978), and guanine to xanthine (Eritja et al., 1986; Kamiya et al., 1992b).

The strongest evidence in support of the deamination mechanism of DNA damage is probably the direct observation of deamination products following treatment of cells or naked DNA with nitric oxide in the presence of oxygen. Mutations caused by nitric oxide treatment of *S. typhimurium* appear to be associated largely with conversion of 5-methylcytosine to thymine (Wink et al., 1991). However, a later study argued against the deamination of cytosine or 5-methylcytosine as the mechanism of nitric oxide mutagenicity (Schmutte et. al., 1994). Nitric oxide-induced mutations in a human lymphoblastoid cell line (TK6) were accompanied by the formation of xanthine and hypoxanthine that presumably arose via deamination of guanine and adenine, respectively (Nguyen et al., 1992). Mutations induced by saturated aqueous nitric oxide in the pSP189 *supF* gene in human and *E. coli* cells found a majority of AT→GC transitions followed by GC→AT mutations (Routledge et al., 1993). The method of $NO^{\cdot}$ delivery seem to influence the type of DNA transitions observed as noted by Routledge et al., 1994. The same plasmid pSP189 at pH 7.4 exposed to $NO^{\cdot}$ donor drugs revealed GC→AT transitions as the most abundant mutation. By contrast, in previous work AT→GC transitions predominated when nitric oxide gas was bubbled through the plasmid solution under otherwise identical conditions.

Initial site-specific mutagenic studies gave further support of xanthine and hypoxanthine giving rise to the above suspected mutations. Kamiya et al. (1992a) studied deoxyinosine in the codon 61 of c-Ha-*ras* gene. Deoxyinosine containing oligonucleotides were transfected into NIH3T3 cells and the DNA was amplified by PCR. The study revealed a predominance of A→G transitions after analyses of 20 clones. Using the same technique, Kamiya et al. (1992b)

constructed a synthetic c-Ha-*ras* gene with deoxyxanthosine positioned in codon 12. The results showed an almost exclusive G→A transition mutational event after analysis of 17 clones. This dissertation proposes a more quantitative site-specific study as further explained in the discussion section.

DNA repair of inosine and xanthine

DNA repair systems in biological organisms are believed to have evolved as a result for the need to reduce mutagenic and toxic lesions induced by DNA damaging agents. Repair mechanisms for the removal of dX and dI are important as explained above. A DNA repair enzyme that specifically removes uracil from DNA, is uracil-glycosylase present in both bacterial and mammalian cells (Lindahl, 1974; Sekiguchi et al 1976; Olsen et al., 1991). Uracil DNA glycosylase has been shown to remove hypoxanthine (Karren and Lindahl 1980); a specific hypoxanthine DNA glycosylase has also been discovered (Karran and Lindahl, 1978), which suggests that hypoxanthine may be a significant genetic threat *in vivo*. There is preferential excision-repair by hypoxanthine-DNA glycosylase of I:T base-pairs which are removed 15-20 times faster than I:C pairs (Dianov and Lindahl, 1991). The characteristics of this excision repair are surprising considering I:C base pairs are the most stable (Aboul-ela and Tinoco Jr, 1985) and would lead to the expected A→G mutation. At present no specific DNA glycosylase has been found for deoxyxanthosine, and this adduct is not removed by hypoxanthine glycosylase (Karren and Lindahl 1980).

Cancer

In tissue undergoing an inflammatory reaction, both the infiltrating and resident cell populations produce a time-dependent radical species that may contribute to DNA damage. Although the flux of radicals per unit time is low, an inflammatory condition that continues for years becomes a significant risk factor for carcinogenic cell transformation. Two organs, stomach and lung are exposed to relevant amounts of exogenous $NO^{\cdot}$ via inhalation of $NO^{\cdot}$ from ambient air and local synthesis in nasopharynx epithelium (Schmidt et al., 1994). The stomach has access to both endogenous (epithelial NOS) and exogenous sources via conversion of nutritional nitrate by facultative anaerobic bacteria to nitrite and could lead to a predisposition for stomach cancer (Esumi and Tannenbaum, 1994). The collection of changes that might be induced in DNA by the mechanisms described in the nitric oxide chemistry section could lead to several types of mutations that are known to be important in human cancer genes. A great deal of attention has been paid recently to the p53 tumor suppressor gene, which is of predominant importance for a number of human cancers including colon, liver, breast and lung (Hollstein et al., 1991).

E. General methods for site-specific mutagenesis: Biological evaluation of a single DNA adduct

As mentioned in the Introduction, site-specific mutagenic studies allow for the measurement of the mutagenic potential or repair of a single DNA lesion. A general description of site-specific mutagenesis is presented (specific details on genome design, construction and xanthine modified pentamer synthesis are presented in the Methods section). Modified oligonucleotides and unmodified controls will be inserted into a derivative of the genome of the bacterial virus M13. Later, specialized vectors will be constructed for evaluation of the mutagenic processing of DNA lesions in prokaryotic cells. The technology for the construction and characterization of site specifically modified genomes was developed in Essigmann laboratory (see Green et al., 1984 and Wood et al., 1990, for typical details). Assembly of the M13 genome to be used for these studies will involve: (1) construction of a five-base gapped heteroduplex genome with complementary DNA bases for a pentamer insert, and (2) complete chemical synthesis of a pentamer sequence containing a single DNA adduct, and (3) ligation of the pentamer with the adduct into the gapped heteroduplex forming a singularly modified genome. This modified genome is then incubated in an *in vitro* system or transfected into a receivable host *in vivo* allowing for biological evaluation (see Figure 6).

Site-specifically situated versus randomly distributed DNA adducts

As mentioned in the oxidative stress section, global modification can not definitively ascribe a specific genetic

event to a specific DNA adduct. Synthetic chemistry and biomolecular techniques have made it possible to construct biologically active singularly modified genomes (Fowler et al.,1982, Green et al., 1984; Loechler et al., 1984; reviewed in Basu and Essigmann, 1988). These tools can be used to address the questions of what effect a specific adduct has on DNA replication and mutagenesis, and what role DNA repair plays in modulating those effects.

Site-specific modification of oligonucleotides

There are several methods for producing a site specifically modified genome and each requires the synthesis of an oligonucleotide which contains a chemically modified nucleotide. The three most common oligonucleotide synthetic strategies are: (1) total synthesis, which involves the production of a modified protected monomer that is used in the solution or solid phase synthesis of oligonucleotide, and (2) chemical or physical modification of a preformed oligonucleotide, and (3) enzymatic synthesis in which DNA or RNA polymerase are used to incorporate modified nucleotides into the oligonucleotide. The total synthetic approach is often preferred because it enables both the facile production of sufficient amounts of the adduct in any desired sequence context. The choice of a method, however, depends on the chemical nature and stability of the adduct to be studied. For example, some adducts are unstable under the conditions required for total synthesis, making this approach unfeasible with current technology. Similarly, the treatment of a preformed unmodified oligonucleotide with a DNA damaging agent may yield products too numerous to allow this approach to be used effectively. The modified

oligonucleotide used for the work presented in this dissertation was prepared by the first method described above. The DNA sequences chosen, and the rationale for their selection, will be presented in the Methods section. The oligonucleotides can be situated in a viral genome context by using one of below mentioned methodologies.

Methods for construction of singular modified genomes

Modified oligonucleotides can be incorporated into the genomes of viruses or plasmids in several ways. In one common method, the oligonucleotide is annealed to the single stranded genome of a viral vector. The 3′ terminus of the oligonucleotide then acts as a primer for the complementary strand synthesis by a DNA polymerase. DNA ligase is used to seal the nick resulting from complete complementary strand synthesis. This method has been used to construct genomes containing site-specifically situated thymine dimers (Huang et al., 1992), psoralen DNA adducts (Kodake and Gamper, 1988). A factor that could limit the usefulness of this approach is secondary structures present in the M13 genomic DNA. M13 DNA assumes secondary structural elements of sufficient stability to prevent efficient replication of the M13 genome (Reckmann et al, 1985). The holoenzyme form of the T4 polymerase can be used to overcome secondary structure problems associated with M13 replication (Kodakek and Gamper, 1988; Szymkowski et al., 1992). Unfortunately the proteins comprising the T4 DNA polymerase holoenzyme are not easily available in their native forms, further limiting the usefulness of this method. An additional drawback of the construction of singly modified genomes by using this approach is the ds nature of the construct produced. M13 ds

genomes are susceptible to strand replication bias effects that prevent a single replication blocking adduct (bulky adduct) from being replicated during DNA synthesis. As a result, M13-derived ss genomes are preferred substrates for site-specific studies of replication blocking lesions and for DNA repair free mutagenic studies.

Another method used for construction of singular modified genomes, a duplex genome is constructed in which one strand contains a gap opposite a sequence that is complementary to that of the modified oligonucleotide to be used. The modified oligonucleotide is annealed to the gapped duplex genome and the nicks on either side of the oligonucleotide are covalently joined by DNA ligase. This method can be adapted to produce ss genomes by engineering a non-ligatable nick in the strand not containing the adduct. The nicked strand can by removed by heat denaturation. This technique was adopted in the genome engineering scheme presented in this thesis. A third technique involves the digestion and removal of the hairpin region of the M13mp7L2 ss genome with Ecori I, followed by annealing and ligation of the modified oligonucleotide into a gap formed with the aid of a scaffold oligonucleotide (Banerjee et al., 1988; LeClerc et al., 1991).

F. M13 bacteriophage and the *lacZ'* gene mutation assay

The vector to be used in the mutagenic study of the single lesion is the M13 system developed by Messing (1983) and adapted for use in site-specifically modified genome construction by C. Lawrence and coworkers (Banerjee et al., 1988; LeClerc et al., 1991). The following is a short description of the biology of the bacteriophage M13 and of the relevant modifications made in the phage genome to facilitate its use as a cloning vector (Meyer and Geider, 1982; Messing, 1983; Zinder and Horiuchi, 1985) and vehicle for the *lacZ'* mutation assay.

M13 life cycle and replication

M13 is a ss F-specific filamentous bacteriophage that infects *E. coli* harboring a F factor plasmid. After infection, the rod shaped phage adsorbs to the F pilus of the host and the coat proteins are removed as the circular strand of the DNA enters the cell. The phage DNA is the template for synthesis of the complementary DNA (-) strand by the host cell replication apparatus, producing a double stranded replicative form (RF) molecule. The M13 RF serves as a template for replication, by a rolling circle mechanism (Figure 7) and for transcription of the mRNA species encoding viral proteins. The protein product of the M13 gene II is required for rolling circle replication. It cleaves the (+) strand at a specific site, and the 3' terminus thereby produced then acts as the primer for replication by the host cell machinery. The 5' end of the (+) strand is displaced as the apparatus moves around the (-) strand template. Once the replication apparatus has returned to the origin, termination occurs when the

displaced (+) strand is cleaved and its 5′ and 3′ ends subsequently are ligated to form a genome-length ss circle. The double stranded RF is sealed and supercoiled, and can again serve as a substrate for gene II and the replication apparatus. Early in infection, the (+) strands formed are replicated to produce RF molecules. Later in the infection process (after approximately 15-20 min), the cellular levels of the product of viral gene V, a single stranded DNA binding protein, are high enough that all of the newly displaced (+) strands are sequestered by the protein, and are thus unable to enter the replication cycle. In this way a steady state level of 100-200 molecules of RF are maintained in the cell. The gene V protein-coated (+) stands are translocated to the cell membrane, where the viral coat proteins are embedded and the DNA is packaged and finally excluded from the cell. M13 phage does not lyse its host. The plaques seen when the phage are plated are actually areas of slowly growing infected cells. Because the M13 bacteriophage is not packaged into preformed capsids, but rather are encapsidated as it emerges from the cell, DNA of almost any length can be packaged. The M13 genomes also contain a small intergenic "polylinker cloning" region into which DNA can be inserted without adverse effects to phage viability. Messing (1983) took advantage of the characteristics of this region in his development of a series of M13 molecular cloning vectors.

Strand bias effects

The viability of bacteriophage M13 genomes in the ds form could lead to a phenomenon known as mutational strand bias. Strand bias effects arise from the ability of either strand of M13 to give rise to progeny independent of the other

strand. As a consequence, a replication blocking lesion in either strand can render the strand inactive and direct the formation of all progeny from the other undamaged strand. A replication blocking lesion in a ds M13 genome could not be replicated and bypassed and its mutagenic effects would not be exhibited. This liability can be avoided by constructing a ss genome containing the DNA lesion; with no undamaged strand present to serve as a template for replication, the adducts must be replicated from the M13 genome to be viable. Also ss genomes maximize potential mutation detection by reducing or eliminating DNA repair.

The M13 *lacZ'* forward mutation assay

Forward mutational assays in non-essential genes have the ability to detect a wide arrange of single-base substitutions, as well as other classes of mutagenic agents such as insertions and deletions at a large number of sites. A forward mutational assay is essential in order to detect subtle difference that might exist in the mutation spectra of similar compounds. The *lacZ'* forward mutational assay utilized in this study has been employed by others to examine the mutagenicity of a variety of DNA damaging agents including UV irradiation (Le Clerc and Istock, 1982; Le Clerc et al., 1984), singlet oxygen (Decuyper-Debeergh et al., 1989), oxygen radicals (Reid and Loeb, 1992), aflatoxin (Sahasrabudhe et al., 1989), 4-aminobiphenyl (Lasko et al., 1988), and apurinic/apyrimidinic (AP) sites (Kunkel, 1984). A survey of these studies revealed that the *lacZ'* forward mutational assay is a sensitive and broad target for the detection of mutants, with 141 single-base substitutions detected at 98 different sites along with additional insertions and deletions.

Molecular basis of *lacZ'* mutational assay

The *lacZ'* forward mutational assay is based on a series of proteins that have β-galactosidase activity. Mutants that disrupt the β-galactosidase activity of these proteins are phenotypically identified colorimetrically in the M13 plaque forming assay. Wild-type β-galactosidase encoded by the *lacZ* gene of the *E. coli lac* operon is enzymatically active as a protein that cleaves β-galactosyl bonds (Kalnins et al., 1983). The active β-galactosidease form is a tetramer. Certain strains of *E. coli* are chromosomally deleted of the *lac* operon but carry the F' *lacZ'* M15 episome that codes for production of the M15 protein. M15 is a naturally occurring mutant of β-galactosidase lacking amino acid residue 11-41. The M15 protein exists as a dimer in solution but is unable to form tetrameres and, despite a strong substrate binding site, lacks enzymatic activity (Langely et al., 1975).

The M15 protein attains β-galactosidase activity in a process known as α-complementation. By definition, this process is the restoration of biological activity by the non-covalent interaction of two or more different proteins. In this case, α-complementation involves two polypeptides, an α-acceptor and an α-donor as shown in Figure 8. When the M15 protein functions as the α-acceptor, the α-donor can be any of several peptide fragments containing the amino acids 11-41 that are deleted from M15; for example peptide fragments 3-92 or 3-41 of wild-type β-galactosidase (Welpy et al., 1981a). The β donor utilized in the M13 *lacZ'* mutational assay is supplied by the *lacZ'* polypeptide encoded by the modified M13 bacteriophage genome created by Messing et al. (1983). The *lac* regulatory region and the first 146 amino acid residues of the *lacZ* gene produced by

Hind II restriction of the *E. coli lac* operon DNA was inserted into the major intergenic region of the M13 genome. The peptide coding region has been further elongated by the addition of a polylinker cloning region in the series of M13 derivatives. The M13 *lacZ'* polypeptide fragment, comprised of 164 amino acid residues, is considerably longer than required for the α-complementation process. The *lacZ'* polypeptide can be divided into three regions, the N-terminal overlap, the M15 host deletion, and the C-terminal overlap. It is well established that the overlapping regions are not essential for α-complementation because the polypeptide encoded by these DNA sequences is duplicated in the M-15 α-acceptor protein. This polypeptide chain required to attain β-galactosidase activity can be supplied by either the M15 α-acceptor or the M13 *lacZ'* α-donor polypeptide (Zabin, 1982., Welpy et al., 1981b). The significance of these regions of the M13 *lacZ'* DNA sequence to the mutational assay is that the N and C terminal overlapping regions are not expected to be as sensitive to the detection of mutations as the *lacZ'* DNA sequence coding for amino acids deleted from the M15 α-acceptor protein. In addition, the *lacZ'*s regulatory region is also expected to give rise to detectable mutations. Mutations in the ribosome binding site, for example influence the production of mRNA, resulting in expression of activity ranging from 1-130% of wild-type, depending on the mutation (Guillerez et al., 1991).

Figure 6. General construction and uses of site-specifically modified genomes. Schematic illustration denotes modified oligonucleotide ligation into a gaped heteroduplex and subsequent biological evaluation of the specific lesion under study.

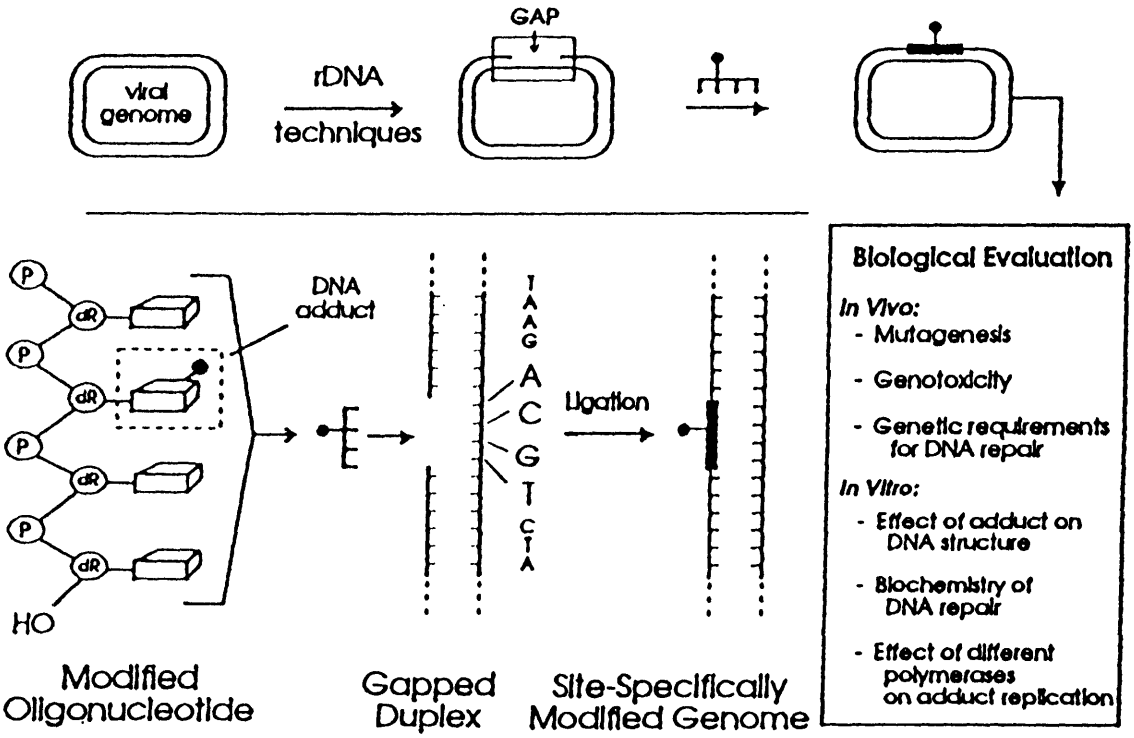

GAP
viral genome
rDNA
techniques
DNA adduct
HO
Ligation
Modified Oligonucleotide
Gapped Duplex
Site-Specifically Modified Genome
Biological Evaluation
In Vivo:
- Mutagenesis
- Genotoxicity
- Genetic requirements for DNA repair
In Vitro:
- Effect of adduct on DNA structure
- Biochemistry of DNA repair
- Effect of different polymerases on adduct replication

Figure 7. Outline of the life cycle of the M13 bacteriophage (from Bradley, 1991). The natural infection of an *E. coli* cell by the single stranded viral form of the M13 bacteriophage genome is shown.

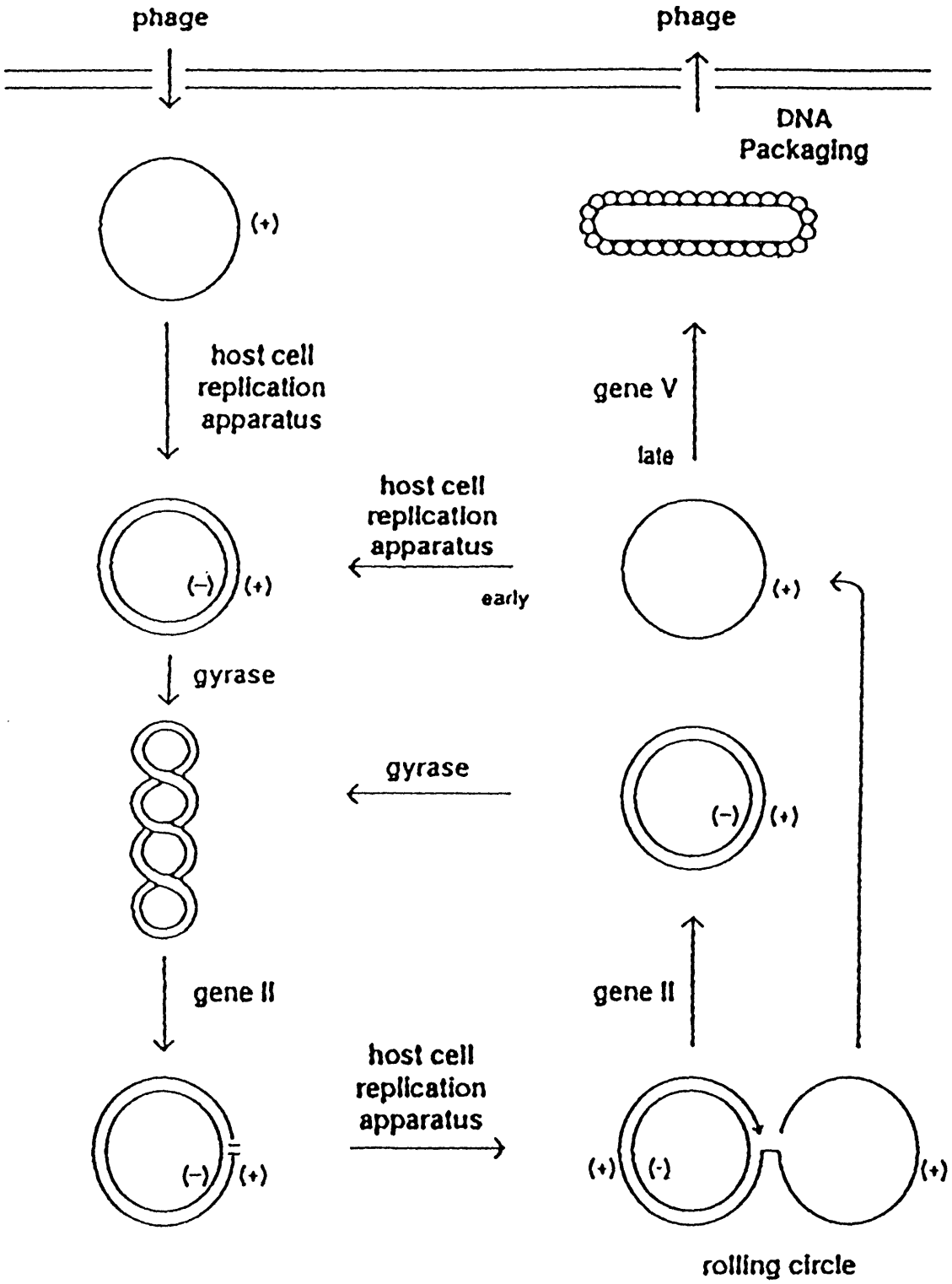
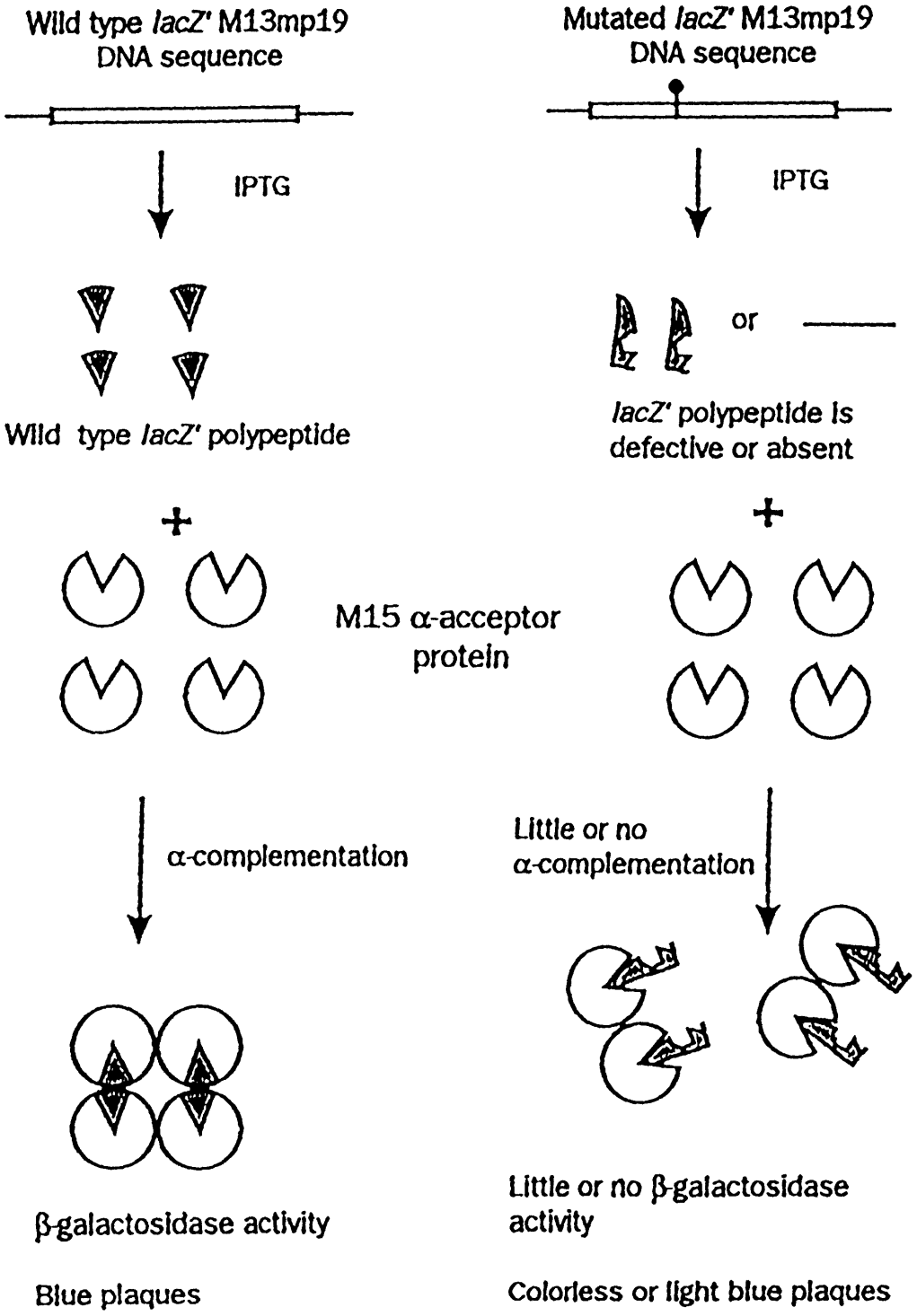

phage
phage
DNA
Packaging
(+)
host cell
replication
apparatus
gene V
late
host cell
replication
apparatus
early
(−)
(+)
(+)
gyrase
gyrase
(−)
(+)
gene II
gene II
host cell
replication
apparatus
(−)
(+)
(+)
(-)
(+)
rolling circle

Figure 8. Molecular basis of the *lacZ'* mutational assay. The *lacZ'* α-complementation process restores β-galactosidase activity to the M15 protein. When complemented with the wild type *lacZ'* polypeptide, the M15 protein tetramerizes and attains β-galactosidase activity that is observed phenotypically by the cleavage of the *X-gal* prochromophore resulting in the formation of blue plaques when plated with *E. coli* GW5100 cells in the plaque forming assay. If the DNA sequence has been mutated, the *lacZ'* polypeptide may not be synthesized (due to a nonsense mutation in the coding region or by disruption of the regulatory region upstream of the coding sequence) resulting in colorless plaques. Alternatively, the *lacZ'* polypeptide may be synthesized, but be functionally compromised, resulting once again in no β-galactosidase activity (colorless plaques) or in some cases diminished activity (light blue plaques). This schematic illustration is a representation of the *lacZ'* mutation assay (Yarema, 1994).


Wild type lacZ' M13mp19
DNA sequence
IPTG
Wild type lacZ' polypeptide
Mutated lacZ' M13mp19
DNA sequence
IPTG
or
lacZ' polypeptide is
defective or absent
M15 α-acceptor
protein
α-complementation
Little or no
α-complementation
β-galactosidase activity
Blue plaques
Little or no β-galactosidase
activity
Colorless or light blue plaques

III. MATERIALS AND METHODS

The experimental system and strategies to be used for the proposed studies can be summarized as: the synthesis of chemically protected deoxyxanthosine, deprotection of deoxyxanthine contained in a pentamer sequence, stability studies of dX or dI modified pentamers, M13 genome design and construction.

A. Materials

Restriction endonucleases (except for PstI and SacI, from Boehringer-Mannhein), T4 polynucleotide kinase and T4 DNA ligase were purchased from New England Biolabs. Phosphatases and DNAses were from Sigma. The *E. coli* cell lines used were: DL7 (AB1157; *lac* U169, *uvr*$^+$, from D. Lasko (1986), MIT) and GW5100 (JM103; P1$^-$, from G. Walker, MIT). Bacterial culture media was from Difco Laboratory. M13mp19 bacteriophage genomes were a gift from J. Messing. Sequencing reagents (Sequenase) were obtained from United States Biochemical Corporation. The oligonucleotides used as sequencing primers were purchased from New England Biolabs or synthesized by the Biopolymers lab, MIT, and purified by high-pressure liquid chromatography.

Chemical reagents used for 2'-deoxyxanthosine synthesis are listed with corresponding vendor in parenthesis: deoxyguanosine (Penninsula Labs, Inc), acetic anhydride (Mallinckrodt), pyridine distilled over calcium hydride, nitrophenolethanol (Aldrich), triphenylphosphine (Aldrich), sodium nitrite (Mallinckrodt), acetic acid (Fisher), acetone (Aldrich), distilled water, dioxane distilled over 4A° molecular sieves, argon (Airco), ammonium hydroxide

(Aldrich), methanol (Mallinckrodt), dimethoxytrityl chloride (Aldrich), methylene chloride distilled over calcium hydride, benzene (Mallinckrodt), bis-diisopropylamine 2-cyanoethyl phosphosamidite (Aldrich) and diisopropylammonium tetrazoldide (Dr. Marjie Solomon).

Other laboratory chemicals and reagents where purchased from Mallinckrodt, with the following exceptions. IPTG and X-gal were purchased from Gold Biotechnology (St. Louis, MO) or Biosynth International (Skokie, IL). DNA purification systems were from the QIAGEN or Promega corporations. Oligodeoxynucleotides used in the construction of xanthine modified genomes were synthesized by Mr. William Kobertz, MIT. All other pentamers were synthesized by using Applied Biosystems reagents and a Model 391 DNA synthesizer PCR mate (Applied Biosystems) or by the Biopolymers Lab, MIT. Semi-preparative HPLC purifications were done on a Waters Associates model 440 detector linked to a Gateway 2000 computer for peak integration and a Beckman 110B solvent delivery model. Analytical HPLC runs deployed a Hewlett Packard model 9133 computer with a 1040A multi-wavelength detection system, Beckman 421A solvent controller and a HP 9000-300 integrator. Ultrafree probind (Millipore) 0.45 μm filter units with modified PVDF membrane for nucleic acid purification were used prior to HPLC injection. Pure oligonucleotides for spectrometry analysis were desalted using a Sep-pak cartridge (Waters Division of Millipore).

B. Preparation of xanthine modified oligonucleotide

Generally complete chemical synthesis of modified nucleosides is the conventional technique used to obtain a single lesion (deoxyxanthosine) in a specific DNA sequence for future mutagenic or repair studies. Although enzymatic synthesis of deoxyxanthosine from mammalian tissues has been described (Friedkin, 1952), only chemical synthesis allows for precise placement of the modified nucleoside into an oligonucleotide. The chemical synthesis of deoxyxanthosine has been achieved (Moschel and Keefer, 1989). However to realize a chemically protected 2'-deoxyxanthosine, two methodologies describing p-nitrophenylethyl protection (Van Aerschot et al., 1987) and 2'-deoxyguanosine oxidation (Eritja el al., 1986) were utilized. The following synthesis of 2'-deoxyxanthosine (see Figure 9.) and subsequent integration of this nucleoside into a pentamer oligonucleotide was accomplished by Mr. William Kobertz.

Chemical synthesis of protected 2'-deoxyxanthosine: 5',3'-o-Diacetyl-deoxyguanosine (1)

Deoxyguanosine (2.08 g, 7.25 mmol) was dissolved in 100 ml of pyridine under argon. Acetic anhydride (20 ml, 212 mmol) was added and the reaction stirred at room temperature for 72 hours. The white glossy precipitate was filtered and washed with warm pyridine and anhydrous ethyl ether. The solid was heated under vacuum overnight to afford 1.75 g (68%) of a flaky white solid.

5',3'-o-Diacetyl-O^6-nitrophenylethyl-deoxyguanosine (2)

5',3'-o-Diacetyl-deoxyguanosine **(1)** (2 g, 5.69 mmol)

nitrophenylethanol (1.43 g, 8.54 mmol) and triphenylphosphine (2.98 g, 11.4 mmol) were dissolved in 100 ml of dioxane under argon. After 15 minutes of stirring, diisopropyl azidocarboxylate (2.25 ml, 11.4 mmol) was added slowly to the cloudy solution which subsequently turned into a clear yellowish color. After 4 hours, the solvent was removed *in vacuo*. The residue was dissolved in 200 ml of CH_2Cl_2. The organic layer was washed with saturated aqueous NH_4Cl (250 ml X 2), dried over anhydrous $MgSO_4$, filtered and the solvent was removed *in vacuo*. Purification of the residue by silica gel chromatography eluting with a gradient of neat CH_2Cl_2 to 50:1 CH_2Cl_2/MeOH afforded a semi-pure yellow solid 3.01 g (106%).

5'3'-o-Diacetyl-O^6-nitrophenylethyl-deoxyxanthosine (3)

5',3'-o-Diacetyl-O^6-nitrophenylethyl-deoxyguanosine **(2)** (1.29 g, 2.87 mmol) was dissolved with 7.5 ml of acetone. A solution of sodium nitrite (5 g) in 15 ml of distilled water was added to the solution. Acetic acid was added to the reaction mixture and was allowed to stir for 2 hours. The cloudy yellowish solution was quenched with saturated bicarbonate and diluted with 100 ml of EtoAc. The aqueous layer was washed with EtoAc (100 ml X 2) and the combined organic layers were dried over $MgSO_4$, filtered and the solvents were removed *in vacuo*. Purification of the solid was accomplished by silica gel chromatography eluting with a gradient (methylene chloride: methanol)(100:1, 50:1, 25:1). The product yield was 0.77 g, a recovery of 53.6%.

5',3'-o-Diacetyl-[(O^2,O^6)-dinitrophenylethyl]-deoxyxanthosine (4)

5'3'-o-Diacetyl-O^6-nitrophenylethyl-deoxyxanthosine **(3)** (256 mg, 0.51 mmol), nitrophenylethanol (129 mg, 0.77 mmol) and triphenylphosphine (268 mg, 1.02 mmol) were dissolved in 10 ml of dioxane. Diisopropyl azidocarboxylate (200 μl, 1.02 mmol) was added to the solution and stirred for 2 hours at room temperature. The dioxane was removed and the resulting semi-solid was dissolved in CH_2Cl_2 and washed once with NH_4Cl. The CH_2Cl_2 layer was dried over $MgSO_4$, filtered and the solvents were removed *in vacuo*. Purification by silica gel chromatography eluting with a gradient of neat CH_2Cl_2 to 100:1 CH_2Cl_2/MeOH afforded a semi-pure yellow solid 375 mg (102%).

5',3'-o-Dihydroxyl-[(O^2,O^6)-dinitrophenylethyl]-deoxyxanthosine (5)

Semipure **(4)** (335 mg, 0.52 mmol) was dissolved in 20 ml of methanol. Ammonium hydroxide (20 ml) was added and allowed to stir overnight. The solvents were evaporated and the remaining white precipitate was dissolved with methylene chloride (200 ml) and was washed with aqueous saturated bicarbonate solution (100 ml). The organic layer was dried over $MgSO_4$, filtered and the solvents were removed *in vacuo*. The crude material was purified by silica gel chromatography eluting with a gradient of 75:1 dichloromethane/methanol to 10:1 CH_2Cl_2/MeOH to afford 199 mg (70.4%) of a white solid. A ^{1}H-NMR of 5',3'-o-dihydroxyl-[(O^2,O^6)-dinitrophenylethyl]-deoxyxanthosine **(5)** is displayed (see figure 10).

5'-Dimethoxytrityl,3'-hydroxyl-o-[(O^2,O^6)-dinitrophenylethyl] deoxyxanthosine (6)

To a solution of 50 mg (0.088 mmol) of compound **(5)** in 160 μl of distilled pyridine were added 33 mg (0.1 mmol) of dimethoxytrityl chloride. The solution was stirred for 6 hours at room temperature then concentrated. Purification of the crude material by silica gel chromatography eluting with a gradient of neat CH_2Cl_2 to 50:1 CH_2Cl_2/MeOH afforded 25 mg (32.7%) of 5'-protected product.

5'-Dimethoxytrityl,3'-phophoamidite-o-[(O^2,O^6)dinitrophenylethyl] deoxyxanthosine (7)

To a solution of 25 mg (0.029 mmol) of compound **(6)** in 250 μl of CH_2Cl_2 were added 4 mg (0.02 mmol) of diisopropylammoniium tetrazoldide (4 mg) and 11 μl (0.035 mmol) of bis-diisopropylamine 2-cyanoethyl phosphate. The solution was stirred under argon for 6 hours. The solvent was evaporated and the residue was purified by silica gel chromatography eluting with 4:1 EtoAc:Hexane to afford 28 mg (91.1%) of product. Mass spectrometry reveal a product weight of 1066 a.m.u. and a molecular formula of $C_{56}H_{59}O_{12}N_8P$.

Incorporation of 5'-dimethoxytrityl, 3'-phophoamidite-o-[(O^2,O^6) dinitrophenylethyl]-deoxyxanthosine (7) into a pentamer (5'-XCAGC-3') oligonucleotide.

5'-dimethoxytrityl,3'-hydroxyl-o-[(O^2,O^6)-dinitrophenylethyl] deoxyxanthosine (7) was dissolved in acetonitrile (250 μl). A DNA synthesizer was used to construct the modified pentamer with a coupling efficiency of 56.9%. Elution of the pentamer from the column was

accomplished with 2 ml of ammonium hydroxide. The residual pentamer was stored at -20 °C subsequent to ammonium hydroxide removal by lyophilization. The elimination of protecting groups from 2'-deoxyxanthosine oligonucleotide was performed by Mr. Esequiel Barrera.

Removal of protection groups from 2'-deoxyxanthosine modified oligonucleotide

The methodology used for removal of protecting groups from 5'-dimethoxytrityl, 3'-phophoamidite-o-[(O^2,O^6) dinitrophenylethyl]-deoxyxanthosine **(7)** incorporated into a pentamer oligonucleotide sequence was described by Eritja et al., 1986.

The white solid **(7)** (250 μg) was added to a solution of 0.5 M of 1,8-diazabicyclo (5.4.0) undec-7-ene (38 μl) dissolved in pyridine (462 μl) under argon. The reaction was incubated for 4 hours at room temperature. After lyophization the yellow-oily solution was passed through a Sephadex G-10 size exclusion column (1.5 cm X 15 cm). The product eluted after 40 ml of water as determined by UV photometric λ_{260} analysis. Speedvac condensation of the product yielded 62.5 μg (24%). The white precipitate was dissolved in water and reacted with concentrated NH_4OH (1 ml) for 16 hours at 65 °C to remove the remaining 5'dimethoxytrityl protection groups and lyophized. Product purification was accomplished by HPLC. Small aliquots of product were saved after each manipulation, and later analyzed by HPLC to monitor reaction progress (see Figure 11).

C. Purification and characterization of synthesized oligonucleotides

All pentamer sequences (5'-XCAGC-3', 5'-GCIGC-3', 5'-GCAGC-3', 5'-GCUGC-3', and 5'-UCAGC-3') were purified by reverse phase HPLC. Initial oligonucleotide separations deployed a Waters HPLC system with a semi-preparatory C18 reverse phase column and a flow rate of 3 ml/min. The gradient solvent system consist of 0-40% of 0.1 M ammonium acetate/acetonitrile for 40 mins followed by steady-state for 10 mins then by 10 mins at 100% acetonitrile. Final oligonucleotide purification was accomplished by using a Hewlett Packard HPLC system with an analytical C18 reverse phase column and a flow rate of 1 ml/min. The solvent system is the same as above except for the gradient of 0-20% of 0.1 M ammonium acetate/ acetonitrile. The HPLC detection limits were set at λ_{210}, λ_{254}, λ_{260} and the threshold at 0.5 mAU. Salt-free oligonucleotide were accomplished with a SepPak cartridge (Waters) eluted with a 50% mixture of MeOH:H2O. Sample recovery is 65% as measured by UV absorption λ_{254}. Oligonucleotide molecular weight were determined by laser deabsorption mass spectrometry, when applicable.

Construction of abasic-site standards

Two HPLC abasic-site containing oligonucleotides (5'-GC*GC-3' and 5'-p*CAGC-3') were constructed. The symbol (*) denotes location of an abasic site. The abasic sites are a consequence of 5-'GCUGC-3' and 5'-pUCAGC-3' incubation with *E. coli* uracil DNA glycosylase (Sigma). The 5' uracil of 5'-UCAGC-3' had to be phosphorylated prior to uracil glycosylase application as noted by Varshney and

van de Sande (1991). Kinase and uracil glycosylase assay conditions are described in Maniatis et al., 1989.

Pentamer stability studies

Initially deoxyxanthosine and later deoxyxanthosine in a pentamer oligonucleotide were tested for stability under conditions necessary for genome construction (Green et al., 1984; Wood et al., 1990). Incubations of modified oligonucleotides (5'-GCIGC-3' and 5'-XCAGC-3') were tested at various temperatures (37 °C, 65 °C, 100 °C) and pH (6.6, 7.0, 7.5, 8.0) of 0.1 M Tris-HCl buffer for durations of 3-30 min. Degradation products were analytically determined by HPLC as noted above.

Enzymatic digestion of modified pentamer sequences

Bovine intestinal mucosa alkaline phosphatase I (1 unit, Sigma), snake venom phosphodiesterase I (2.5 units, Sigma) and pure oligonucleotides (2 μg, 1.42 nmole) were added to 0.1 M Tris-HCl, 5 mM $MgCl_2$ (pH 8.5) and incubated for 4 hours at 37 °C. Afterwards the solution was filtered with a 0.45 μm probind cartridge and microcentrifuged for 2 min. Individual nucleosides were determined using a C18 reverse-phase analytical column (Beckman), Hewlett Packard HPLC system and a flow rate of 1 ml/min. Calibrated nucleoside standards were used to help identify digested modified oligonucleotides. All nucleosides are commercially available except for deoxyxanthosine, which was synthesized by Mr. William Kobertz. The same solvent system as above was used with exception of the programmed gradient: 0-20% of 0.1 M ammonium acetate/acetonitrile (60 min), followed by steady-state for (5 min) then 20-100% of acetonitrile

(10 min). Nucleoside extinction coefficients values (Maniatis et al., 1989) are: 11.7 ml/μmole (dG), 7.3 ml/μmole (dC), 15.4 ml/μmole (dA), 11.4 ml/μmole, (dX, Moschel and Keefer, 1989) and 12.3 ml/μmole, (dI, *Specifications and criteria for biochemical compounds*, 1967).

D. Bacteriophage and host bacteria

DL7 *E. coli* cells is the host cell for transfection of the M13mp19 phage. This is an excision repair-competent strain that does not contain a functional lactose operon. This cell strain is particularly useful due to low color background when X-gal indicator is used. *E. coli* GW5100 cells are used as the indicator strain for the M13mp19 phage transfected host cell. The GW5100 strain contain the F′ episome that is required by the M13 phage for infection. M13 phage secreted by the transfected host cells can infect the surrounding GW5100 cells, thus forming plaques. GW5100 cultures require the complementary portion of the *lacZ′* α operon provided by the M13mp19 to create a functional lactose operon. Details are described in the M13 Experimental System section and references therein.

E. Preparation and design of modified M13 genomes

M13 precursors necessary for gapped heteroduplex construction

M13 precursors were given by Ms. Debbie Kruetzer and Dr. Dipti Mathur. The precursor construction deployed the Mutagene M13 in vitro mutagenesis kit (Bio-Rad laboratories) and methodology described by Kunkel et al., 1987. The (+)

single-stranded uracil-containing DNA template was isolated by growing recombinant bacteriophage M13mp19 in a *dut*$^-$, *ung*$^-$ F'$^+$ *E. coli* cell strain with a uracil rich solution. The *E. coli dut*$^-$, *ung*$^-$ mutant lacks the enzymes dUTPase and uracil DNA-glycosylase, respectively. The precursor construction entails a two step manipulation deploying two primer sequences.

41 mer: 5'-GGGATCCTCTAGAGTCGACC**G**TGCAGGCATGCAAGCTTGGC-3'
45 mer: 5'-AACGACGGCCAGTGAATTCG**GCTGC**AGCTCGGTACCCGGGGATCC-3'

Purification of 41 mer and 45 mer primers was accomplished by a running a 12.5% polyacrylamide gel. Both primers are complementary to the (+) single-strand of the M13mp19 genome except for 1 and 5 base areas denoted in bold which give rise to small "bumps". The bumps are specifically located opposite unique PstI and SacI restriction site of the *lacZ'* α gene. Initially the phosphorylated 41 mer is annealed to the ss uracil-containing (+) DNA strand. Subsequent completion of the (-) strand is accomplished by T4 DNA polymerase, dNTPs and T4 DNA ligase. Transfection of isolated ds phage DNA into an *E. coli* (*ung*$^+$, *dut*$^+$) strain destroy the uracil-containing (+) strand forcing replication from the (-) strand. The resulting progeny has a single base insert at the PstI site, named the M13mp19+1 genome. Using the M13mp19+1 genome as a template, the same methodology is repeated using the 45 mer. The subsequent five-base insertion at the SacI site forms the genome M13mp19+6 (see Figure 12).

M13mp19+1 and M13mp19+6 characterization

RF phage DNA (1 μg) was treated with PstI or SacI

restriction enzymes and separated by 0.8% agarose gel electrophoresis. RF DNA that showed the desired characteristics (19+1, PstI resistance) and (19+6, SacI resistance) were selected for further amplification after DNA sequence verification. The mutant amplified RF population was retransformed into DL7 cells and the progeny was used to infect GW5100 cells to produce a second RF population as noted below.

Amplification of M13 precursor genomes

Overnight GW5100 cell culture (100 μl), 2x YT media (1 ml) and a single plaque were incubated for 6 hours on a rotatory drum at 37 °C. An additional liter of 2x YT media was added to the GW5100/phage culture and incubated for 8 hours. The culture was put on ice for 30 min and centrifuged (7000 RPM, 10 min). The supernatant contained the ss phage and the precipitate held the RF DNA. The supernatant was further treated with 29.2 g of NaCl/liter and 40 g PEG 8000 and stirred at 4 °C for 30 min. Centrifugation (10,000 RPM, 30 min) allowed for ss phage pellet formation. Small-scale RF DNA extractions (phage miniprep kit, Promega) coupled with an 0.8% agarose gel electrophoresis were used to detect miniphage presence. Cultures containing miniphage were discarded. Large scale RF or ss phage DNA isolation was accomplished with a Maxi-prep DNA purification kit (Qiagen) or by phenol extraction (Maniatis et al., 1989). Purity of the DNA was verified by monitoring A_{260}/A_{280} ratios and by running aliquots on a 0.8% agarose gel and straining with ethidium bromide. Electrophoretic conditions were set at 100 volts with constant current and a 2 hour run time. RF DNA to be used for heteroduplex construction required removal of minor contaminants consisting of nicked, linear,

and ss DNA species. RF DNA contaminants were separated after centrifugation using a 5-20% sucrose density gradient (see Maniatis et al., 1989) and analyzed by gel-electrophoresis as noted earlier. RF DNA/sucrose fractions were collected and the sucrose removed by microdialysis at 4 °C (dialysis membrane, Gibco-BRL, catalogue no. 1202MF). Pure RF DNA was obtained after ethanol precipitation.

Transfection and plating of progeny phage

Overnight cultures of GW5100 (grown in 2x YT media) or DL7 (grown in LB media) cells were allowed to reach confluence. An aliquot of DL7 (2 ml) overnight culture was used to commence a fresh DL7 cell culture (250 mL) until the log phase growth of 90-100 Klett units was obtained. The standard plating assay is described by Messing, (1983). Phage ss DNA (200 ng per sample) was mixed with about 2 X 10^9 transformation ($CaCl_2$) competent DL7 cells and left on ice for 60 min. Afterwards the samples were heat-shocked (42 °C) for 1.5 min and chilled on ice again for an additional 1.5 min. Serial dilutions of transfected cells were add to a mixture of overnight GW5100 cells (300 μl), isopropyl-β-D-thiogalactoside (10 μl, [24 mg/ml]), 5-Bromo-4-chloro-3-indoly-β-D-galactopyranoside (40 μl, [40 mg/ml] in dimethylformamide), and 2.5 ml of melted agar (42 °C) then immediately plated on B-broth agar plates. Plaque and color development occurs in 1 to 2 days. The presence of a mutant bacteriophage can be detected by the colorless or light blue plaque as compared to the normal dark blue color of wild type plaques.

Construction of heteroduplex genome with a five base gap

As a result of base insertions, the M13mp19+1 genome has an out of reading frame destroyed PstI site and gives rise to colorless plaques. The M13mp19+6 genome has a restored reading frame with regained PstI susceptibility and yields dark blue plaques. A similar case is noted by Wood et al. (1990). The gapped heteroduplex construction uses 50% mixtures of M13mp19+1 SacI linearized DNA and M13mp19+6 BglII linearized DNA. The BglII restricted DNA is further treated with calf intestinal phosphatase (Sigma) to remove 5′ phosphates this prevents self-religation. The two linearized genomes are heat denatured and allowed to hybridize (see Figure 13). The resulting five base gapped heteroduplex is engineered to accept the ligation of the generic oligomer sequence 5′-GCAGC-3′. A second gapped molecule could also form but will pose no complication for the reasons presented by Wood et al. (1990).

Pentamer sequence flexibility for gapped heteroduplex ligation

The pentamer sequence was carefully design to carry out site-specific mutagenic studies of deoxyxanthosine, deoxyinosine and a host of other possible mutagenic lesions (see Figure 14). The pentamer sequence is complementary to the five-base gap of the heteroduplex.

```
3'.....CTCGACGTCGG.....5' (-) strand of M13mp19+6
5'.....GAGCTGCAGCC.....3' (+) strand of M13mp19+1
           XCAGC
           GCIGC
```

The underlined area denotes the generic pentamer ligated to

the five-base gap. The modified pentamer sequences (5′-pXCAGC-3′ and 5′-pGCIGC-3′) are designated to also ligate to above gap. The first position of the 5′ end of the generic pentamer sequence was chosen as the site for deoxyxanthosine incorporation. This location allowed for less chemical exposure of the deoxyxanthosine-phosphoramidite as the oligonucleotide was chemically synthesized 3′→5′. The fourth position of the same pentamer sequence is another suitable location for deoxyxanthosine. The pentamer sequence carrying a single oxidative lesion is 5′ phosphorylated and later ligated into the gapped heteroduplex forming a singularly modified ds genome. The non-ligatable nicked (-) strand is heat degraded leaving a ss modified (+) genome ready for cell transfection and biological evaluation (see Figure 15).

DNA sequencing of mutants

The dideoxynucleotide chain termination DNA sequencing method (Sanger et al., 1977) was used to sequence the *lacZ′* α gene, a 380 base pair region from position 6100-6480. Reagents were provided by a DNA sequencing kit (Sequenase, version 2, United States Biochemical Corporation). Two short single stranded primers were annealed to the M13 phage DNA, one at a site adjacent to the *lacZ′* α region and one within the gene. The following primers were used: P6331 AAG TTG GGT AAC GCC and P6527 GTT TGA GGG GAG GAC. These primers are complementary to the circular ss DNA and the above number designates the 5′ nucleotide position to which the primer anneals in the M13mp19 genome. The complementary strand synthesis is done by DNA polymerase in the presence of the four deoxynucleotide triphosphates (dNTPs). The incorporation of the ddNTPs terminates the synthesis of the

strand and result in double stranded DNA products of different lengths. After heating these products briefly at 95 °C in formamide, the reactions are loaded onto adjacent lanes of a denaturing 6% acrylamide gel. The presence of 7 M urea prevents complementary strands from reannealing to each other during electrophoresis. The ^{35}S labeled fragments are detected by autoradiography and the sequence deduced from the band pattern. The gel was dried and exposed to X-ray film for 1-5 days at room temperature.

Figure 9. Precursors of chemically protected 2'-deoxyxanthosine. The p-nitrophenol ethyl protecting group (NPE) is linked to the sixth position of guanine. Subsequently, the amino group of guanine is deaminated and oxidized to a carbonyl group. The phosphoramidite deriative is formed and incorporated into the pentamer with the NPE group still covalently attached. Further chemical deprotection, purification and characterization was carried out prior to oligonucleotide stability studies.

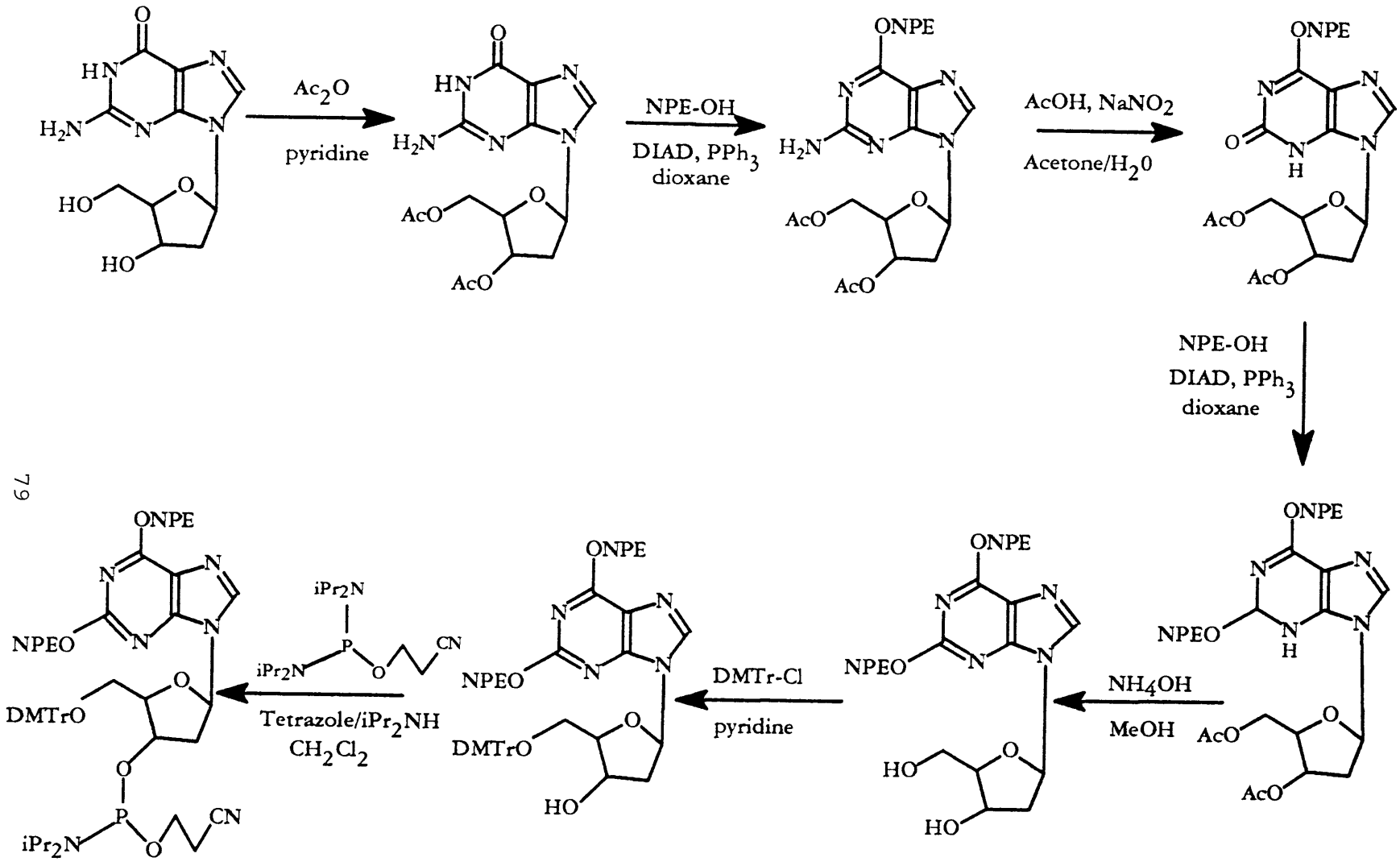

Ac2O
pyridine
NPE-OH
DIAD, PPh3
dioxane
AcOH, NaNO2
Acetone/H20
NPE-OH
DIAD, PPh3
dioxane
NH4OH
MeOH
DMTr-Cl
pyridine
Tetrazole/iPr2NH
CH2Cl2
ONPE
NPEO
DMTrO
AcO
HO
CN
iPr2N

Figure 10. Proton nuclear magnetic resonance spectrum of diprotected deoxyxanthosine. After NMR characterization, fully and partial deprotected xanthine nucleoside where later used to provide nucleoside standards to monitor chemical deprotection treatment. These standards also revealed that xanthine in an oligonucleotide contains a stable glycosidic bond under conditions necessary for genome construction.

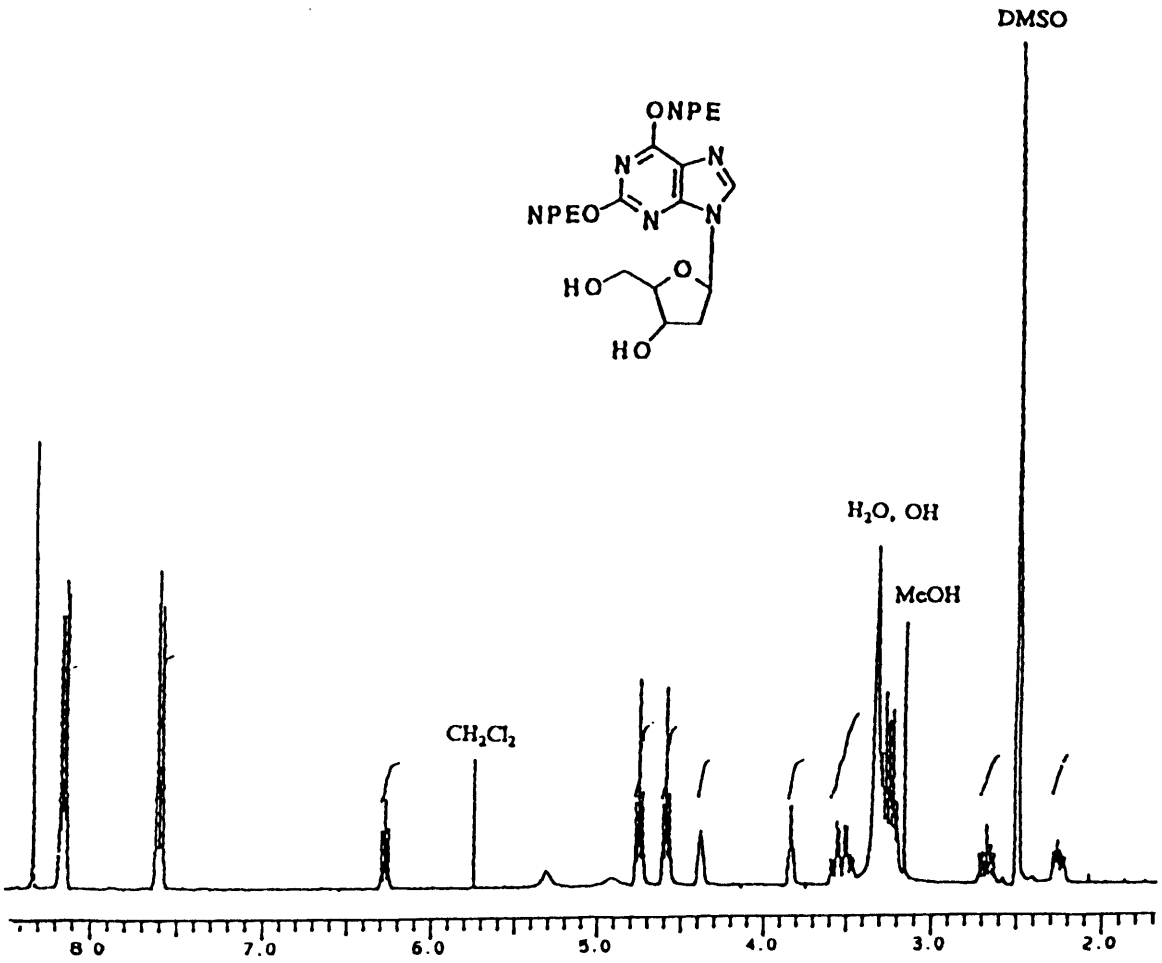

DMSO
ONPE
N
N
NPEO
N
N
HO
O
HO
H2O, OH
MeOH
CH2Cl2
8.0
7.0
6.0
5.0
4.0
3.0
2.0

Figure 11. Deprotection flow chart of xanthine containing oligonucleotide. Removal of p-nitrophenyl ethyl (NPE) protecting groups were accomplished in a series of chemical steps. After each chemical manipulation small aliquots were saved and subsequently analyzed by high pressure liquid chromatography and ultra violet spectroscopy. This process served as reaction monitors of the chemical deprotection series. Ultimately, the deprotected oligonucleotides were purified, characterized and used for stability studies of the oligonucleotide under chemical conditions necessary for genome construction.

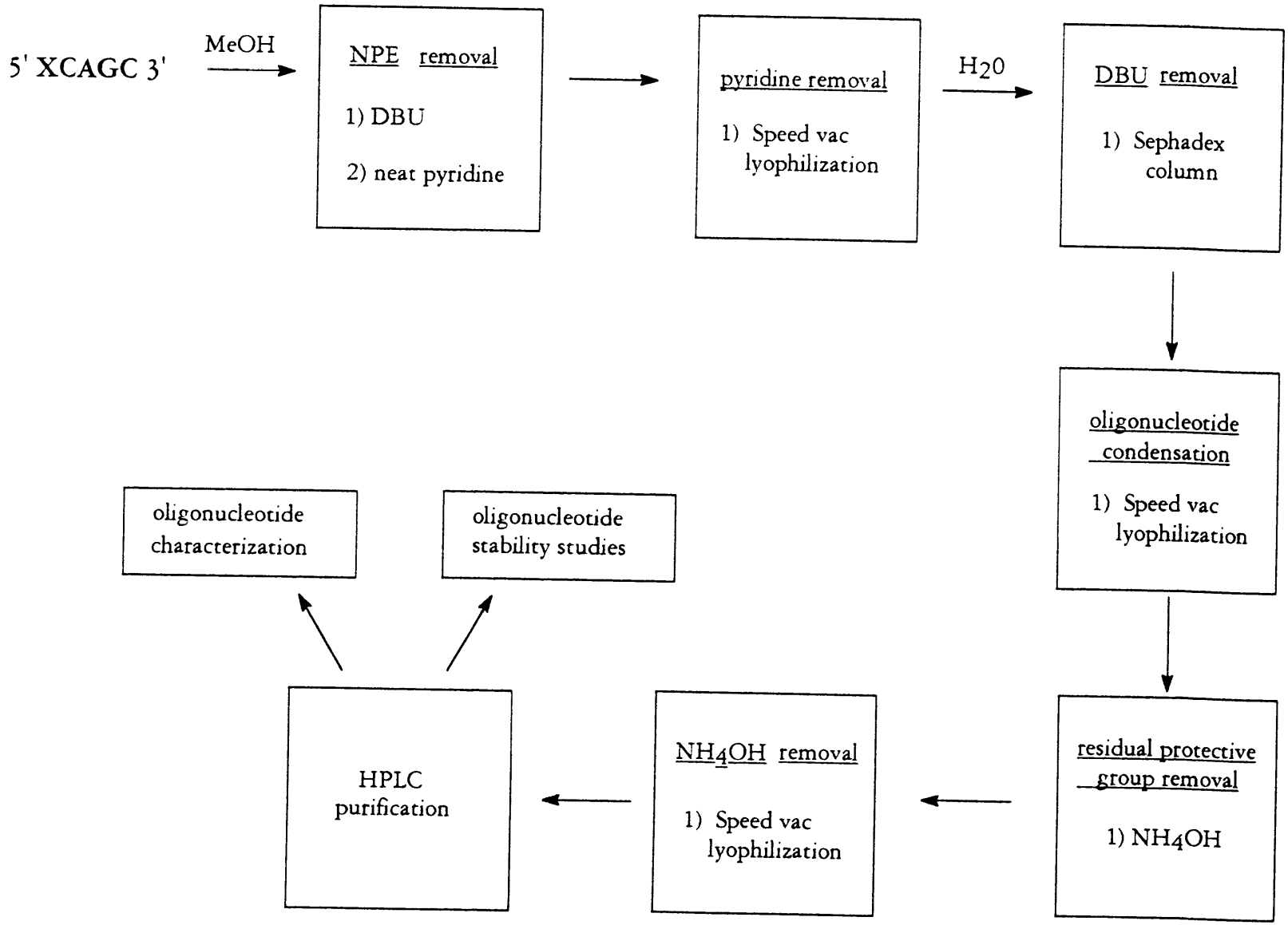

5' XCAGC 3'
MeOH
NPE removal
1) DBU
2) neat pyridine
pyridine removal
1) Speed vac lyophilization
H20
DBU removal
1) Sephadex column
oligonucleotide condensation
1) Speed vac lyophilization
residual protective group removal
1) NH4OH
NH4OH removal
1) Speed vac lyophilization
HPLC purification
oligonucleotide characterization
oligonucleotide stability studies

Figure 12. Recombinant DNA construction of gapped heteroduplex precursors. Wild type M13mp19 was modified by insertion of a cytosine at a PstI site in the *LacZ'* gene by standard genetic techniques. The newly added nucleotide forms the M13mp19+1 genome and is refractory to PstI digestion. The M13mp19+1 serves as a precursor for the further addition of five bases at a SacI site downstream of the initial singular insertion. This genome is noted as M13mp19+6 and regains a functional PstI site although subsequently refractory to SacI digestion. Both the 19+1 and 19+6 genomes are employed for gapped heteroduplex construction.

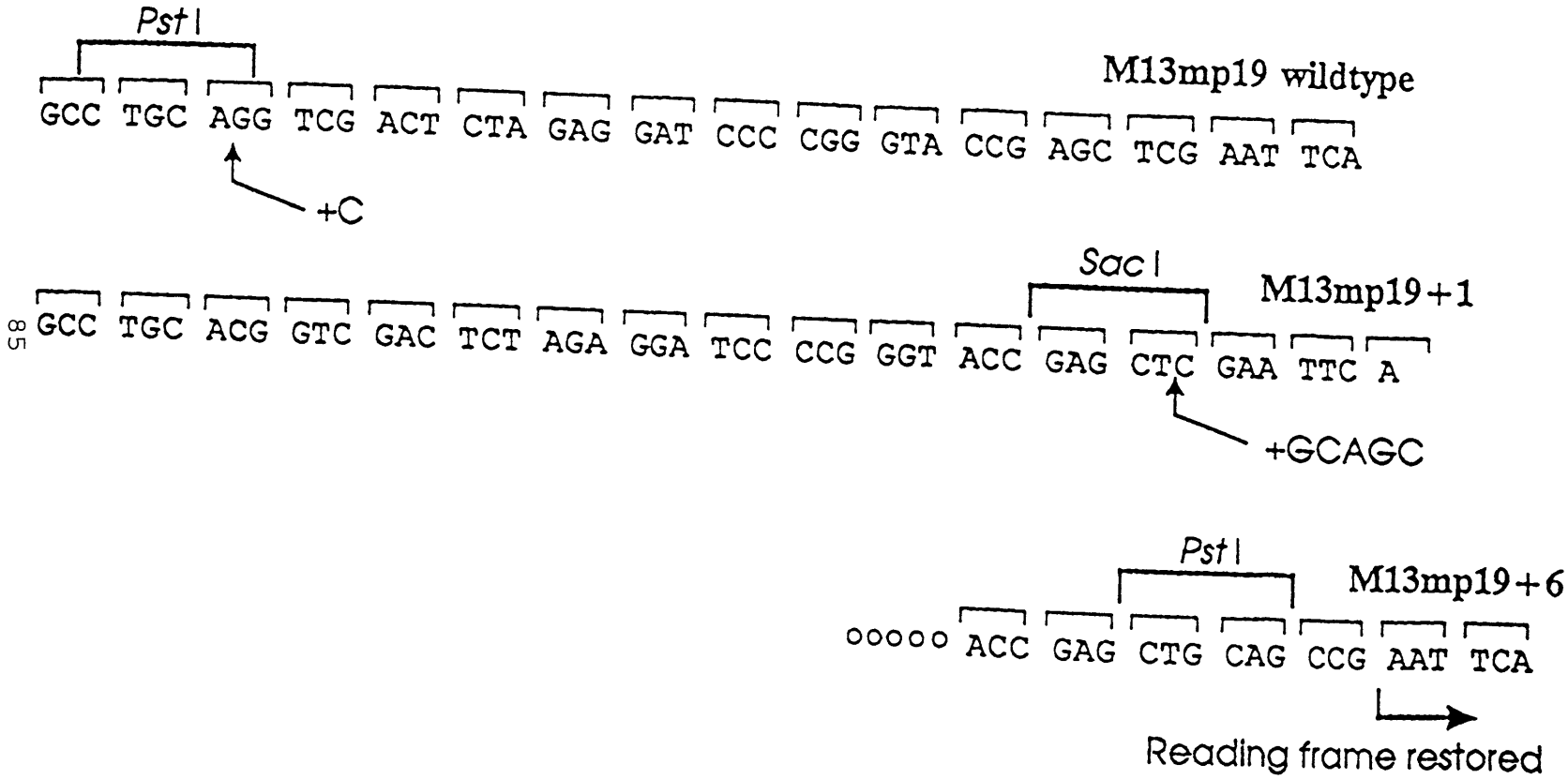

Pst I
M13mp19 wildtype
GCC TGC AGG TCG ACT CTA GAG GAT CCC CGG GTA CCG AGC TCG AAT TCA
+C
Sac I
M13mp19+1
GCC TGC ACG GTC GAC TCT AGA GGA TCC CCG GGT ACC GAG CTC GAA TTC A
+GCAGC
Pst I
M13mp19+6
ooooo ACC GAG CTG CAG CCG AAT TCA
Reading frame restored

Figure 13. Specific details in the construction of the gapped heteroduplex genome. The M13mp19+1 was linearized with SacI restriction and mixed with M13mp19+6 linearized with BglII and dephosphorylated. Calf intestinal phosphodiesterase (CIP) removes the phosphate at the 5′ end of the DNA strand which effectively inhibits oligonucleotide self-religation. Heteroduplex formation was accomplished by heating and cooling with fifty percent mixtures of M13mp19+1 and M13mp19+6.

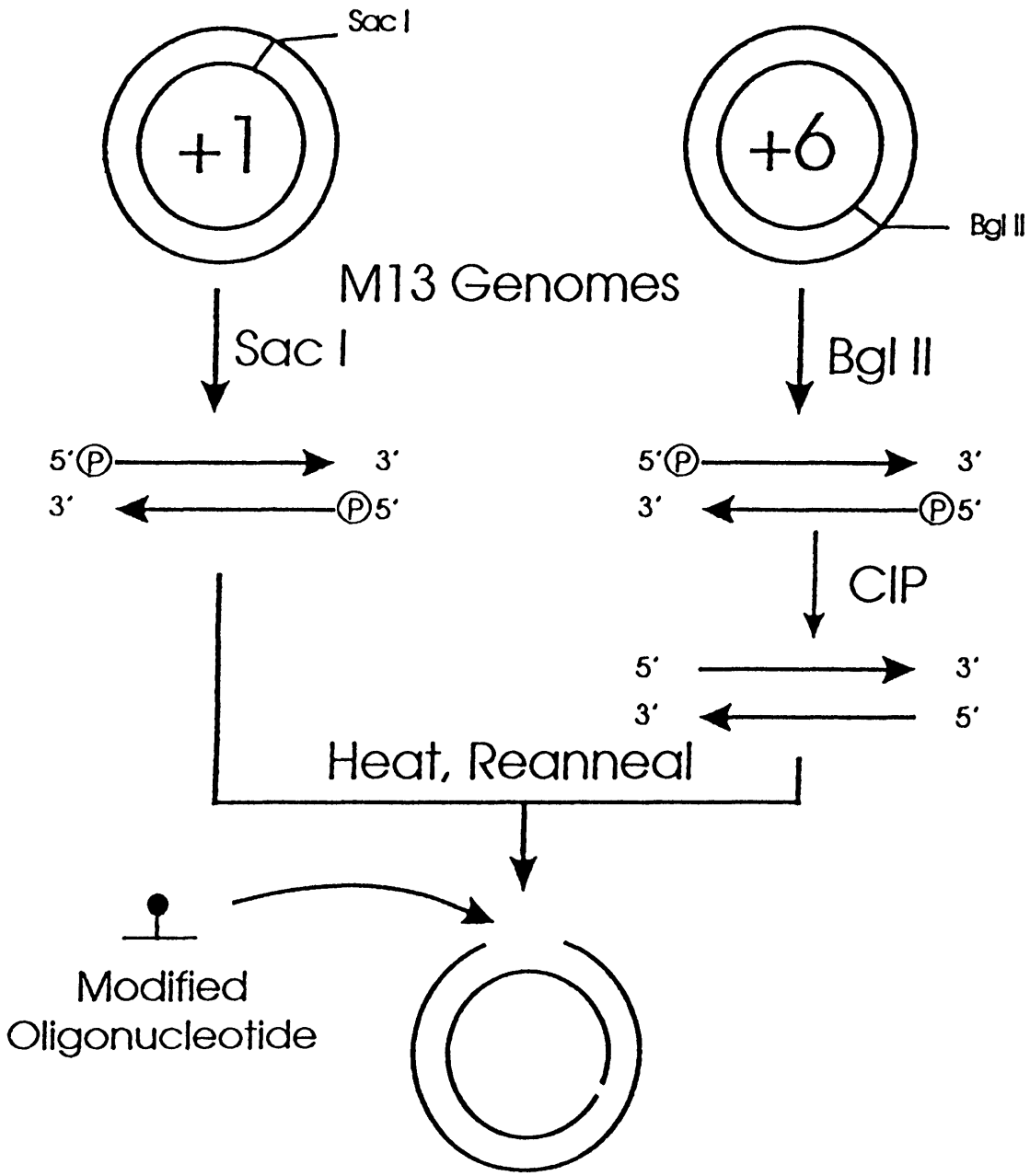

Sac I
+1
+6
Bgl II
M13 Genomes
Sac I
Bgl II
5'Ⓟ
3'
3'
Ⓟ5'
CIP
5'
3'
3'
5'
Heat, Reanneal
Modified
Oligonucleotide

Figure 14. Modified pentamer sequence carrying various oxidative lesions. The gapped heteroduplex is complementary to the pentamer insert carrying the modified nucleotide. This study focuses on xanthine (X) and hypoxanthine (Hx) modified oligonucleotides. However, other nucleotide sites on the pentamer can be used to measure the genotoxicity of other oxidative lesions. In the figure, M13-NO· denotes the gapped heteroduplex formation.

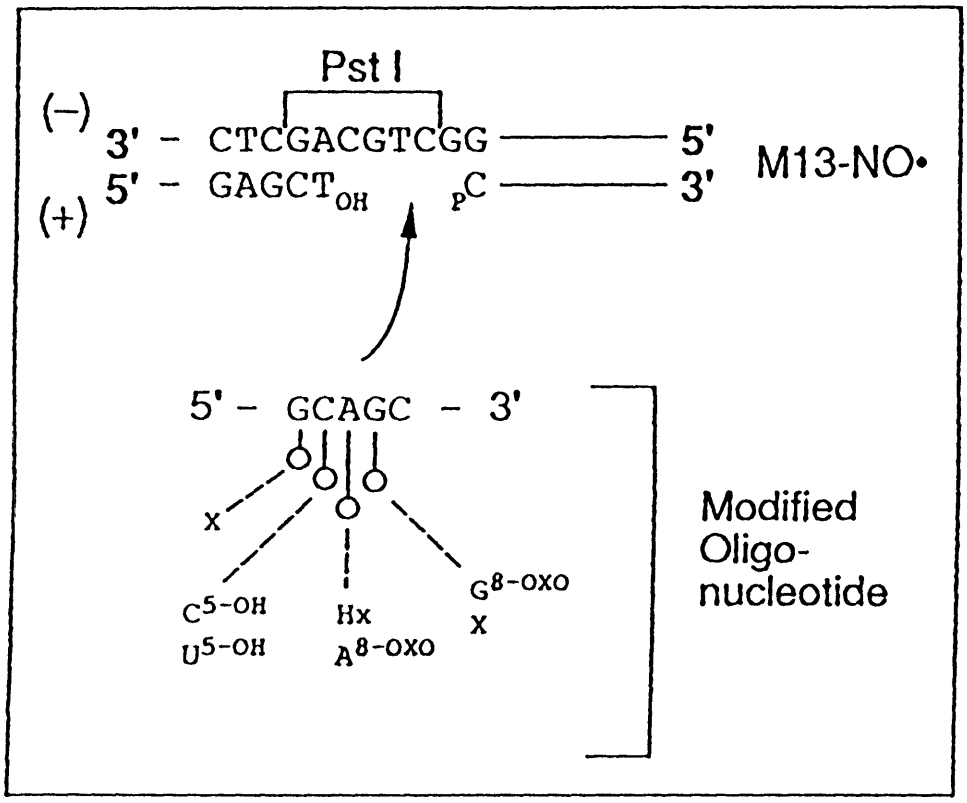
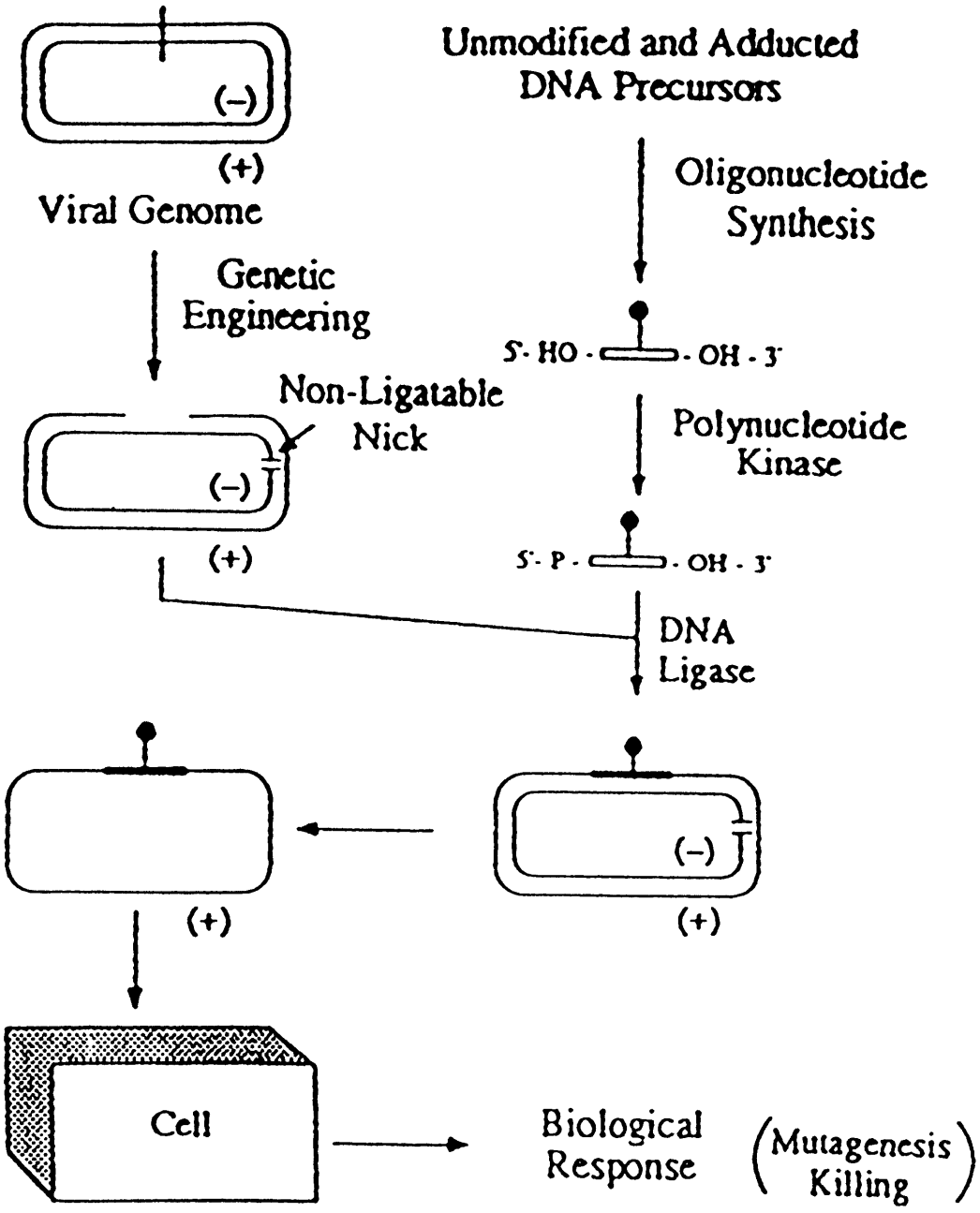

Pst I
(–)
3' - CTCGACGTCGG —— 5'
M13-NO•
5' - GAGCT$_{OH}$ $_{P}$C —— 3'
(+)
5' - GCAGC - 3'
X
C^{5-OH}
U^{5-OH}
Hx
A^{8-OXO}
G^{8-OXO}
X
Modified
Oligo-
nucleotide

Figure 15. Construction of single stranded M13 genome containing a single lesion. The positive sign (+) in the figure denotes the M13mp19+1 genome and the negative symbol (-)denotes the M13mp19+6. The five base gap formed in the heteroduplex is formed by the union of plus and negative strands. This gap allows for complementary base pairing of the pentamer carrying a modified nucleoside to be placed directly in the gap. Prior to genome transfection into *E. coli*, the minus strand not containing the modified nucleoside is removed. The remaining modified, single stranded genome allows for mutagenic measurement of the lesion independent of repair mechanism present in *E. coli*.


(–)
(+)
Viral Genome
Genetic
Engineering
Non-Ligatable
Nick
(–)
(+)
Unmodified and Adducted
DNA Precursors
Oligonucleotide
Synthesis
5'- HO - OH - 3'
Polynucleotide
Kinase
5'- P - OH - 3'
DNA
Ligase
(–)
(+)
(+)
Cell
Biological
Response
Mutagenesis
Killing

IV. Results

HPLC analysis after xanthine-containing pentamer deprotection noted 8.5% abasic site and 33% 5′-XCAGC-3′ of the total reaction mixture. The retention time difference between both species is 0.8 min (Table 1). Finer separation of the 5′-XCAGC-3′ from the AP species of 5′-XCAGC-3′ typically yield 65% of amount injected. The digestion of modified oligonucleotides (Figure 16) and comparison with controls (Table 2) confirm the expected nucleoside ratios and complete removal of 5′-XCAGC-3′ protecting groups. Distinctive UV spectra of nucleosides (Figure 17) helped determine the various nucleoside species. The identification of the 5′-XCAGC-3′ AP species was verified by the absence of deoxyxanthosine. Further analysis of the 5′-GCIGC-3′ by mass spectrometry was found to yield a molecular weight of 1488 a.m.u. (Figure 18).

Both modified pentamers (5′-XCAGC-3′ and 5′-GCIGC-3′) are stable for conditions necessary for genome construction (Wood et al., 1990). Specifically, the 5′-XCAGC-3′ pentamer showed only a single HPLC peak (no signs of degradation) under heating conditions of 100 °C for 3.5 min. A separate study conducted at the same temperature for 3 min using deoxyxanthosine was also stable but incubation for 5 min showed signs of product degradation. The 5′-XCAGC-3′ under kinase reaction conditions (37 °C, 30 min, 65 °C, 15 min, pH 7.8) demonstrated a stable product however a minute shoulder was detectable. The unidentified shoulder represents less than 1% of the HPLC peak area. The potential problem of readily labile glycosidic N-4′ to C-1′ links in deoxyxanthine containing oligonucleotides, particularly in

acidic environments, was not observed in conditions required for genome fabrication. The 5′-GCIGC-3′ is a very stable species from pH 6.6-7.5 and no evidence of instability was noted at 100 °C.

Using genetic engineering techniques, a five-base gapped heteroduplex genome was constructed. The gap position was made complementary to various single-lesion containing pentamer sequences. Possible mutations that arise from deoxyxanthosine (X) and deoxyinosine (I) are devised to convey progeny PstI resistance. Specifically, the PstI recognition site (in bold, showing only one strand), 5′...**CTGCAG**C...3′ contains the described 5′-GCAGC-3′ sequence. Premutagenic sequences 5′-XCAGC-3′ and 5′-GCIGC-3′ could give rise to G→A and A→G transitions, respectively, which change the PstI recognition sequence (underlined) to 5′...CTACAGC...3′ and 5′...CTGCGGC...3′. This sequence change makes the PstI site non-functional. Using the above characteristic, spontaneous DNA mutations not associated with the specific lesion region (PstI site) can be removed by rounds of PstI restriction. Attributes of the *lacZ′* gene mutation assay expresses both spontaneous and lesion-induced mutations. Therefore, enrichment of mutant progeny by PstI restriction ensures more quantitative results. The M13 genome design and mutation selection scheme presented in this thesis will facilitate the future site-specific mutagenic studies of deoxyxanthosine and deoxyinosine *in vivo*.

Table 1. HPLC retention marks of various pentamer sequences. Stability of oligonucleotides was analyzed under various pH (6-8) and temperatures (100 °C, 3.5 min, 37 °C, 15 min) necessary for genome construction. The following symbol (*) denotes an abasic site location in the pentamer sequence.

HPLC RETENTION MARKS

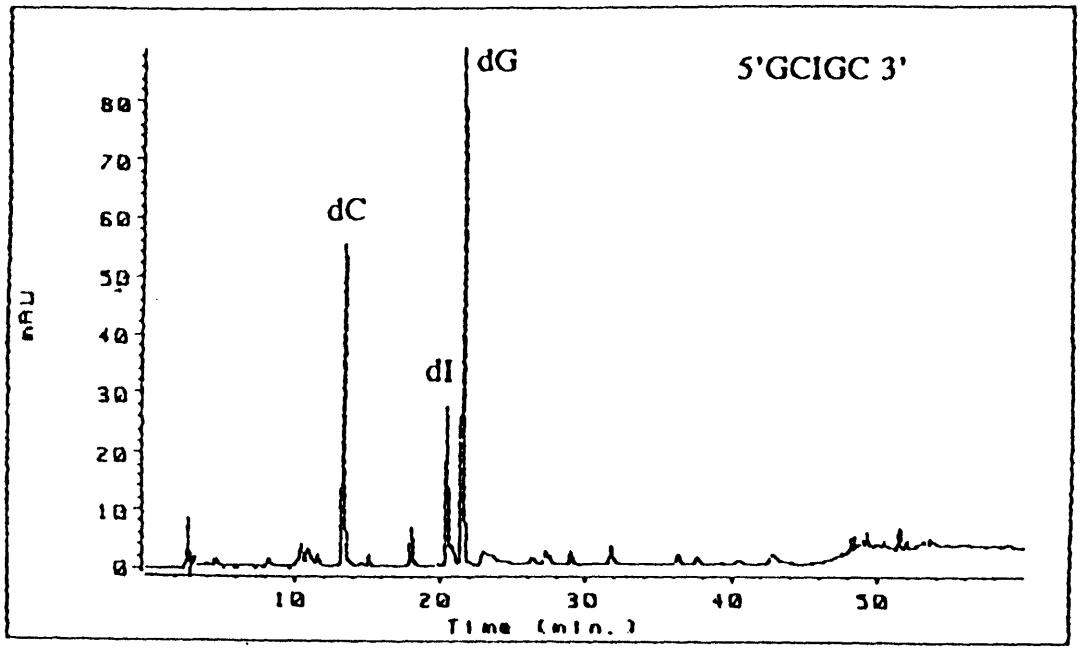
GRADIENT 0-40% 0.1 M AMMONIUM ACETATE:ACETONITRILE

SAMPLE	(MINS)
5′-GCAGC-3′ (semi-prep)	29.7
5′-GCAGC-3′ (analytical)	24.5
5′-GCAGC-3′ (pH stability)	25.6
5′-GCAGC-3′ (genome stability)	25.1
5′-GCIGC-3′ (semi-prep)	31.4
5′-GCIGC-3′ (analytical)	24.6
5′-GCIGC-3′ (pH stability)	24.3
5′-GCIGC-3′ (genome stability)	24.2
5′-GCUGC-3′ (semi-prep)	30.9
5′-GCUGC-3′ (analytical)	25.8
5′-GC*GC-3′ (abasic site)	23.5
5′-UCAGC-3′ (semi-prep)	35.8
5′-UCAGC-3′ (analytical)	23.3
5′-UCAGC-3′ (5′hydroxyl)	27.8
5′-UCAGC-3′ (5′phosphate)	23.2
5′-*CAGC-3′ (abasic site)	20.2
5′-XCAGC-3′ (abasic site, semi-prep)	28.7
5′-XCAGC-3′ (semi-prep)	29.5

GRADIENT 0-20% 0.1 M AMMONIUM ACETATE:ACETONITRILE

5′-XCAGC-3′ (abasic site, analytical)	29.5
5′-XCAGC-3′ (analytical)	30.3
5′-XCAGC-3′ (genome stability)	30.1

Figure 16. Enzymatic digestion of modified pentamers containing either deoxyinosine (dI) or deoxyxanthosine (dX). Cytosine and xanthine have a similar retention time but distinctive ultraviolet spectra. This difference allowed for further characterization of this pentamer. All digested nucleoside ratios are consistent with expected values as compared to quantified standards.


dG
5'GCIGC 3'
dC
dI
mAU
80
70
60
50
40
30
20
10
0
10
20
30
40
50
Time (min.)

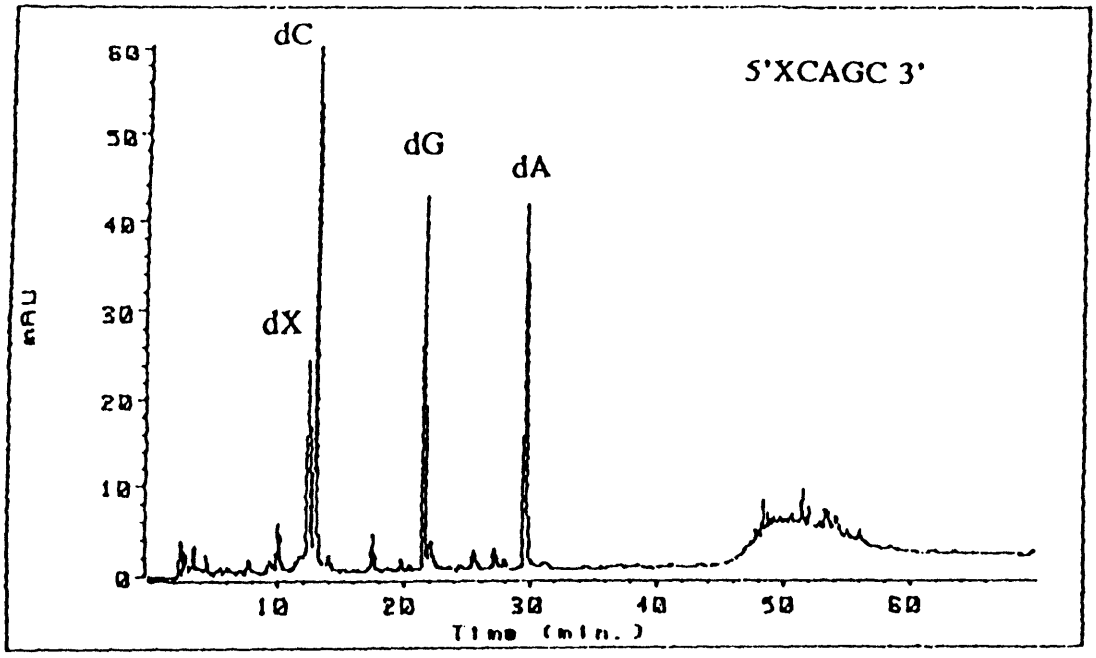

dC
5'XCAGC 3'
dG
dA
dX
mAU
60
50
40
30
20
10
0
10
20
30
40
50
60
Time (min.)

Table 2. HPLC retention marks for enzymatic digestion of pentamers and nucleosides standards. HPLC retention marks where obtained using an analytical high pressure liquid reverse-phase (C18) column. A 0-20% solvent gradients of 0.1 molar mixture of ammonium acetate to acetonitrile was used to achieve resolution over a run time of 75 min.

OLIGONUCLEOTIDE ENZYMATIC DIGESTS

HPLC RETENTION TIMES

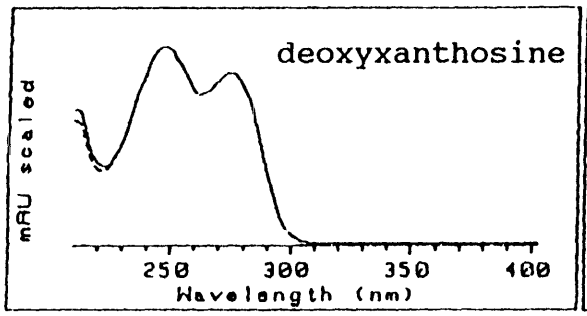
GRADIENT 0-20% 0.1 M AMMONIUM ACETATE:ACETONITRILE

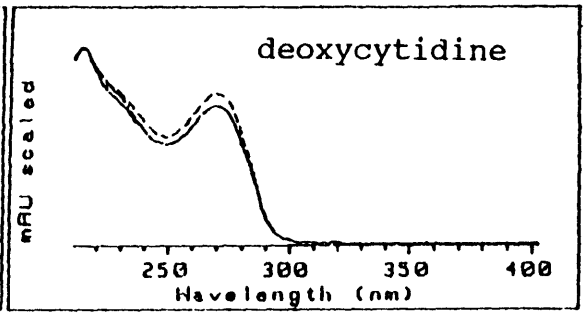
SAMPLE	**(MINS)**
5'-GCAGC-3' (undigested)	30.1
5'-GCAGC-3' (digested)	
dA	29.2
dC	13.6
dG	21.7
5'-GCIGC-3' (undigested)	29.6
dC	13.6
dG	21.7
dI	20.7
5'-GCAGC-3' (control, digested)	
dA	35.7
dC	14.3
dG	25.5
5'-XCAGC-3' (abasic site undigested)	29.7
5'-XCAGC-3' (abasic site undigested)	
dA	36.1
dC	14.5
dG	25.8
5'-XCAGC-3' (undigested)	29.8
5'-XCAGC-3' (digested)	
dA	36.1
dC	14.5
dG	25.7
dX	14.0

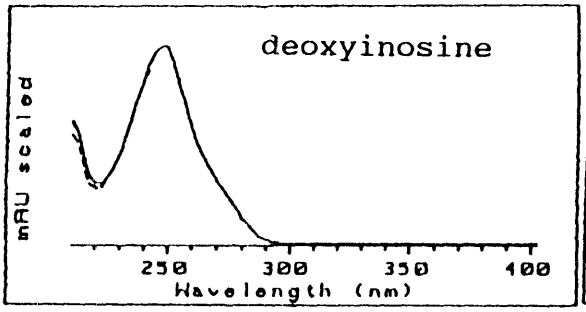
SYNTHESIZED STANDARDS

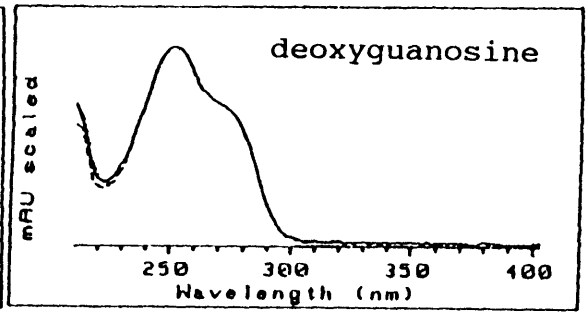
DEOXYXANTHOSINE	14.1
DEOXYXANTHOSINE (MONOPROTECTED)	51.7
DEOXYXANTHOSINE (DIPROTECTED)	
PEAK #1	53.9
PEAK #2	56.3

Figure 17. The ultra violet spectra of nucleosides following enzymatic digestion. Each spectrum is arranged according to retention time as eluted from a C18 reverse phase HPLC column. The y-axis denotes milli-absorbance units (mAU) and the x-axis is the wavelength of light in nanometers (nm). All spectra were taken using a deuterium light source and multi-wave detector set at 210 nm, 254 nm and 260 nm.


deoxyxanthosine
mAU scaled
250
300
350
400
Wavelength (nm)


deoxycytidine
mAU scaled
250
300
350
400
Wavelength (nm)


deoxyinosine
mAU scaled
250
300
350
400
Wavelength (nm)


deoxyguanosine
mAU scaled
250
300
350
400
Wavelength (nm)

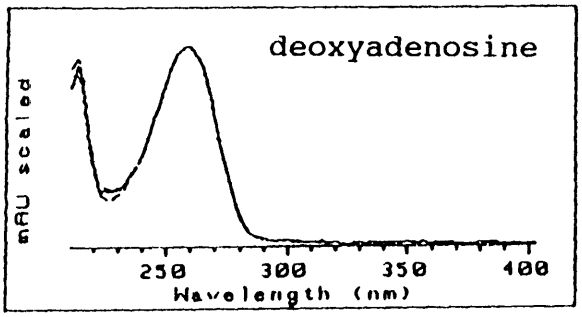
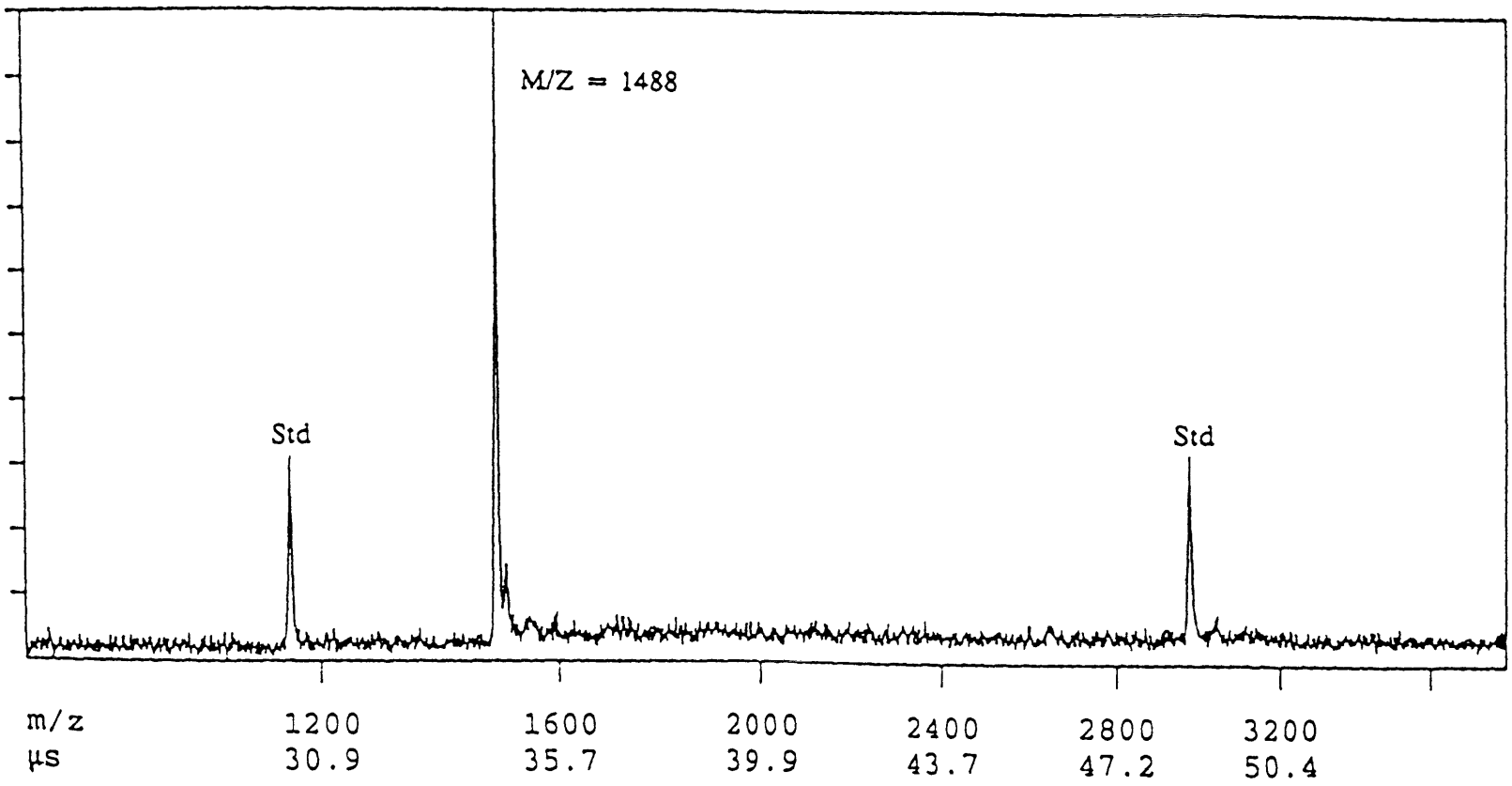

deoxyadenosine
mAU scaled
250
300
350
400
Wavelength (nm)

Figure 18. Laser deabsorption mass spectrometry of modified penatmer containing deoxyinosine. The oligonucleotide retains a molecular weight of 1488 atomic mass units.


M/Z = 1488
Std
Std
m/z
µs
1200
30.9
1600
35.7
2000
39.9
2400
43.7
2800
47.2
3200
50.4

V. Discussion and future research

Site-specifically modified DNA oligonucleotides are convenient models for probing the chemical and biological effects of individual adducts (Basu and Essigmann, 1988). The diversity of the adduct population following the exposure of DNA to oxidants, nitric oxide or ionizing radiation is likely to be the cause of the many different types of mutations induced in genomes of mammalian (Grosovsky et al., 1988; Nguyen et al., 1992), bacterial (Glickman et al., 1980), and viral origin (Ayaki et al., 1986; Hoebee et al., 1988). The mutational spectra observed in these assay systems show a preference for single base substitutions, the majority of which tend to be transitions or transversions at GC pairs (Glickman et al., 1980; Ayaki et al., 1986; Tindall et al., 1988; Hoebee et al., 1988; Hayes et al., 1988; Moraes et al., 1989). Although much work has been done on oxidative lesions there is uncertainty as to which lesions give rise to the predominant G:C→A:T, GC→TA and GC→CG mutations. The molecular lesion(s) giving rise to the GC→TA transition could arise from the deamination of an unstable oxidation product of cytosine (Hayes et al., 1988). This hypothesis is being further investigated by the Essigmann lab with the specific candidates: 5-hydroxycytosine, 5-hydroxyuracil and uracil glycol. Another possibility to account for the observed G:C→A:T transition is purine deamination leading to the formation of xanthine in DNA (Wink et al., 1991; Nguyen et al., 1992, Kamiya et al., 1992b).

By using site-specific mutagenesis techniques as described in this thesis, deoxyinosine, deoxyxanthosine and the above cytosine derivatives could be studied in parallel. By using

the same genetic system and pentamer sequence, conclusions on the relative mutability of the various lesions can be reached. Site-specific mutagenesis will allow us to rank the mutagenic significance of each lesion studied. Results from these studies combined with analytical information of the amounts of each adduct present in cellular DNA, will provide the basis for an estimate of the relative genetic risks of the lesions formed by nitric oxide and oxygen radicals. As mentioned earlier, site-specific mutagenesis studies have been reported for hypoxanthine (Hill-Perkins et al., 1986, Kamiya et al., 1992a) and xanthine (Kamiya et al., 1992b). In the two latter works, only the qualitative features of mutagenesis were reported (sequenced clones $\leq$ 20). The proposed site-specific mutagenic experiment will be a statistically more extensive study. The possible mutations from the above base deaminations will give rise to an *amber* codon enabling facile phenotypic detection of mutants by their color on an indicator agar. Gross changes in sequence (e,g., targeted deletions) affecting the original adduct site are detectable but untargeted mutations remote from the adduct site would probably be missed; such mutations are expected to be rare.

Other future investigations of oxidative and nitric oxide mutagenesis could deploy *E. coli* strains with different DNA replication and repair backgrounds as demonstrated by Basu et al., 1989. The influence of the inducible *E. coli* SOS system on mutagenesis could be also examined (Lasko et al., 1988). These studies are aimed at determining whether the DNA lesions under investigation are mutagenic and, if so, whether there are specific genetic requirements for that mutagenesis. These studies would order the mutagenic potential of DNA lesions in wild type cells. Additionally,

through the use of a mutant host, they also define the host genes that effect these mutations, and the genes that protect the cell against mutagenesis. The described lesions showing mutagenic activity in bacterial cells will be tested for mutagenesis in a mammalian site-specific mutagenesis assay (Ellison et al., 1989). Observations of site-specific mutagenic studies along with potential animal models containing for example the *lacZ'* gene (Douglas et al., 1994) are useful tools for *in vivo* detection and quantification of induced gene mutation.

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