MUTAGENIC CONSEQUENCES OF REPLICATION BYPASS OF N3 URACIL AND N^2 GUANINE DNA ADDUCTS

by

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CERTIFICATE OF APPROVAL

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LIST OF ABBREVIATIONS

ABS	acrylonitrile-butadien-styrene plastic
ALDH2	mitochondrial aldehyde dehydrogenase
AO	alkenal/one oxireductase
BER	base excision repair
BD	butadiene
BDM	butadiene monomer
BD N3-dU	butadiene-derived N3 2′deoxyuridine
BSA	bovine serum albumin
CGE	capillary gel electrophoresis
CPD	cyclobutane pyrimidine dimers
CZE	capillary zone electrophoresis
dA	deoxyadenosine
dC	deoxycytosine
dG	deoxyguanosine
dI	deoxyinosine
DEB	1,2:3,4-diepoxybutane
DHN	1,4-dihydroxy-2-nonene
DMT-Cl	Dimethoxytrityl chloride
DMT-dU-Bu-OSi	5′-O-DMT-2′-deoxyuridine-N3-(2-O-tBDMSi-3-butene)

DMT-dU-BuOSi-CE	5′-O-DMT-2′-deoxyuridine-N3-(2-O-tBDMSi-3-butene)-3′-O-(O-cyanoethyl-N,N-diisopropyl)-phosphoramidite
DMT-dU-(Bu-OSi)-Tol	5′-O-DMT-3′-O-toluoyl -N3-(2-O-tBDMSi-3-butene) -2′-deoxyuridine
DMT-dU	5′-O-Dimethoxytrityl-2′-deoxyuridine
DMT-dU-Tol	5′-O-DMT-3′-O-toluoyl-2′-deoxyuridine
dRP	Deoxyribosephosphate
DSB	double-stranded break
EB	1,2-epoxy-3-butene
EBD	3,4-epoxy-1,2-butanediol
EH	epoxide hydrolase
4HAA	4-hydroxynonanal
HB Val	N-(2-hydroxy-3-butenyl) valine
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
His	histidine
HMVK	hydroxymethylvinylketone
4HNA	4-hydroxynon-2-enoic acid
HNE	trans-4-hydroxynonenal
HNE-dG	1, N^2 deoxyguanosine adducts of 4-hydroxynonenal
α-HOPdG	α-hydroxy-1,N^2-propano-2′-deoxyguanosine

β-HOEdG	1,N^2 β-hydroxyethanodeoxyguanosine
γ-HOPdG	γ-hydroxy-1,N^2-propano-2′-deoxguanosine
HPLC	high perfomance liquid chromatography
ICL	interstrand crosslink
IPTG	isopropylthio-β-galactoside
Kf	Klenow fragment
LHC	lymphohaemotopoietic cancer
MALDI-TOF	matrix assisted laser desorption/ionization-time of flight
α-Me-γ-HOPdG	α-methylated-γ-hydroxypropanodeoxyguanosine
m^6G	O^6-methylguanine
MMR	mismatch repair
MNNG	N-methyl-N′-nitro-N-nitrosoguanine
MS	mass spectrometry
MTD	maximum tolerable limit
NA	not available
NER	nucleotide excision repair
NMR	nuclear magnetic resonance
N1-THBAde	N1-(2,3,4-trihydroxybutyl) adenine
8-oxoG	7,8-dihydro-8-oxoguanine
PAHs	polycyclic aromatic hydrocarbons
PCNA	proliferating cell nuclear antigen

Pol	polymerase
(6-4) PPs	6-4 photoproducts
Rf	relative front
RT	room temperature
SB latex	styrene-butadiene latex
SBR	styrene-butadiene rubber
SHM	somatic hypermutation
Ss	single-stranded
tBDMSi	tert-butyldimethylsilyl
THB Val	N-(2,3,4-trihydroxybutyl) valine
TK	thymidine kinase
TLC	thin-layer chromatography
TLS	translesion synthesis
XPV	xeroderma pigmentosum

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ABSTRACT

DNA is subject to attack from various endogenous and exogenous chemicals to yield DNA adducts and these alterations represent an important step in the pathway to mutagenesis and carcinogenesis. This work focuses on the mutagenicity of three different DNA adducts that have been introduced into DNA using site-specific approaches. These adducts include the butadiene-derived N3 2′deoxyuridine (BD N3-dU) adducts, the 1, N^2 deoxyguanosine adducts of 4-hydroxynonenal (HNE-dG), the 1, N^2 β-hydroxyethano deoxyguanosine (β-HOEdG) adduct derived from vinyl chloride and the α*R* and α*S* methylated γ-hydroxypropano deoxyguanosine (α-Me-γ-HOPdG) derived from crotonaldehyde. These adducts were selected due to their highly stable nature and high toxicity of the parent compounds from which they are derived. These adducts were synthesized into oligodeoxynucleotides and replicated through mammalian cells using a single-stranded shuttle vector. Due to their differential base pairing properties, these lesions lead to error-prone replication. Replication past the BD N3-dU adducts was found to be ~97% with a high percentage of C to T transitions and C to A transversions. However, they were highly blocking to Klenow Fragment of DNA polymerase I, pol epsilon, pol delta and pol beta. A survey of translesional polymerases revealed that pol eta could efficiently incorporate opposite the adducts and extend past them although both incorporation and extension required more than one encounter with the polymerase. Pol zeta was also found to extend past the adducts. Additionally, pol eta and pol zeta act synergistically to bring about the bypass of the BD N3-dU adducts. The potential to initiate base excision repair of these adducts was also evaluated using base excision

repair glycosylases UDG, SMUG1 and Endo III; however, none of these were able to excise the BD N3-dU adducts. The HNE-dG adducts were differentially mutagenic in that the (*6R, 8S, 11R*) and (*6S, 8R, 11S*) HNE-dG adducts induced at least 4-fold greater point mutations than the (*6R, 8S, 11S*) and (*6S, 8R, 11R*) stereoisomers, with the overall mutation rate being less than 5%. Replication past the β-HOEdG was found to be 2% mutagenic. In the case of the α-Me-γ-HOPdG adducts, replication past these adducts were ~5-6%. In summary, this dissertation has examined the mutagenicity of DNA adducts derived from both endogenous (HNE and crotonaldehyde) and exogenous sources (butadiene and vinyl chloride). Among these adducts, the BD N3-dU adducts were found to be the most mutagenic. Furthermore, this work has identified the translesional polymerases that may be responsible for the mutagenic bypass of these adducts observed in mammalian cells.

CHAPTER 1

INTRODUCTION

General Introduction

Cells are the structural units of all living things. The most fundamental property of cells is the ability to reproduce themselves. In eukaryotic organisms, this is a highly regulated process that ensures orderly production of cells to maintain tissue integrity. Occasionally, a cell acts in an aberrant fashion and divides continuously without attaining maturity. This uncontrolled proliferation of mammalian cells can lead to tumor formation, metastasis, and ultimately death of the organism.

Cancer is caused by mutations in the genetic material of a cell, and it is through multiple mutagenic events that the fine balance between cell proliferation and cell death is disturbed. In fact, a series of mutations in certain classes of genes is required before a normal cell will transform into a cancer cell (*reviewed in* Devereux et al., 1999). Those classes include genes involved in the control of cell proliferation, programmed cell death (apoptosis), and DNA repair. Mutations in DNA may occur spontaneously, or result from damage caused by endogenous or exogenous sources. Substances that cause these mutations are known as mutagens, and mutagens that cause cancers are known as carcinogens. Some examples of well known carcinogens include asbestos, nickel, cadmium, uranium, radon, vinyl chloride, benzidene, and benzene.

Cells have evolved elaborate systems to recognize and repair DNA damage. There are many genes that encode enzymes that recognize specific kinds of damage in DNA, such as those caused by oxidative radicals, radiation, or chemicals. Often gene products work together and form multi-protein complexes that coordinate the repair of DNA damage. These pathways inlude base excision repair (BER), nucleotide excision repair (NER), and mismatch repair. However, sometimes, cells with damaged genomes

escape repair pathways, yet still survive due to a mechanism called as damage tolerance. There are two pathways of damage tolerance: translesion synthesis (TLS) and recombination repair. These are pathways that do not remove the DNA damage, but overcome blocks to DNA replication.

One consequence of industrialization is the exposure of organisms to chemicals not encountered naturally. Overall, the incidence of cancer has risen dramatically. It accounts for 25% of all deaths in the US and developed countries. The percentage of those that die from chemical exposures is difficult to establish as it is a bioaccumulative process and depends on other factors such as lifestyle and diet. Nevertheless, chemical exposure is an important area for research. The work presented in this dissertation takes a close look at several industrial and endogenous chemicals associated with cancer or known to cause DNA damage, and examines the interaction of their resultant DNA adducts with the replication machinery.

Historical Perspective

Chemical carcinogenesis had its origins about three centuries ago when in 1775 an English surgeon Percival Pott observed a high rate of scrotal cancers among chimney sweeps (Pott, 1963). Pott was able to make this association at a time when a causal connection for exposure and disease had not been established for any disease. Pott's insights led to the novel concept that exposure to a specific agent could cause disease. Other examples of exposure-related cancer followed: pipe smoking and lip cancer in 1795, coal tar in 1875, shale oil in 1876, arsenic in the late 1800's, synthetic dyes and bladder cancer in 1895, X-irradiation in 1902, lung cancer and radioactive material from

mining in 1926, bone sarcomas and radium paint in 1929, sunlight in 1937 and mesotheliomas in asbestos workers in 1960. A prominent theory in the 1800's was that cancer was caused by chronic irritation. Yamagiwa and Ichikawa advanced this idea of chronic inflammation causing cancer by showing in 1915 that one could induce skin cancers in rabbits by painting the skin with a solution of coal tar (Yamagiwa and Ichikawa, 1918). Although their proposed mechanism was wrong, this experiment was of considerable importance since it was the first animal carcinogenecity study for chemical carcinogens. Prior to this period in 1910, the carcinogenic effects of radiation were observed in experiments with rats, representing the first successful induction of tumors in animals. The chronic inflammation theory was soon rejected due to several observations. One was that in some mice painted with coal tar, there were no skin cancers but lung cancers. Second, some complex mixtures of hydrocarbons were carcinogenic, while others were not, even though both produced irritation. This then led to attempts to identify what active molecules were present in the complex mixtures of carcinogenic agents such as soot or coal tar. This question was pursued by Kennaway's group in the 1930's. They were able to purify some of the carcinogenic agents, the most potent of which was identified as benzo[a]pyrene, a polycyclic aromatic hydrocarbon and found it to be carcinogenic in mice (Kennaway and Hieger, 1930). In parallel, Cook was able to synthesize several carcinogenic compounds (Cook, 1937). These studies constituted a major advance in the study of cancer. Therefore cancer development was no longer ascribed to a non-specific irritation of the tissue. There were only certain molecules that were carcinogenic suggesting a specificity in the mechanism of action. In 1947, Miller and Miller made a breakthrough in the field by providing the first evidence for the

metabolism of a carcinogen to its active metabolite (Miller and Miller, 1947). By 1969, after several studies were performed with the reactive form of carcinogens it was postulated by Miller and Miller that the reactive forms of the majority of chemical carcinogens are electrophilic reactants that react with nucleophilic groups in macromolecules to initiate carcinogenesis. Another important finding around that time was the multi-stage nature of carcinogenesis (*reviewed in* Miller and Miller, 1979). It can be divided into three different stages, as depicted in Fig 1.1: (1) initiation, where DNA is irreversibly altered; (2) promotion, which is the multiplication of altered cells; and (3) progression, which involves chromosomal changes, high growth rate, invasiveness, and potential to metastasize.

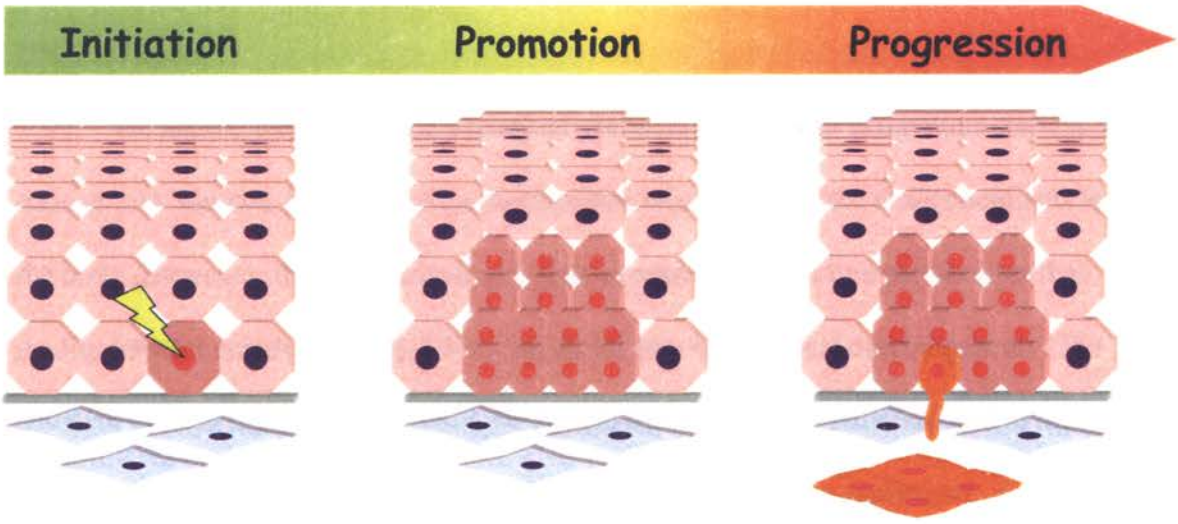


Fig 1.1. Multistage model of carcinogenesis

DNA Adducts

We are exposed to a wide variety of chemicals from both exogenous sources and endogenous sources. Detoxification mechanisms exist to eliminate these chemicals from the body. However, these chemicals can also be converted to active metabolites that readily interact covalently with DNA, the initial step in chemical carcinogenesis. The covalent modification of DNA bases by chemicals can alter the structure and in turn, the biological processing of the DNA by cellular proteins governing replication, transcription and repair. The work presented in this dissertation focuses on four different DNA adducts: the butadiene (BD)-derived N3 2′deoxyuridine (BD N3-dU) adducts, the 1,N^2 deoxyguanosine adducts of 4-hydroxynonenal (HNE-dG), the 1,N^2β-hydroxyethano deoxyguanosine (β-HOEdG) adduct derived from vinyl chloride and the α*R* and α*S* methylated γ-hydroxypropano deoxyguanosine (α-Me-γ-HOPdG) adducts derived from crotonaldehyde.

Butadiene

BD (CH=CH-CH=CH) is a four carbon, doubly unsaturated compound that exists as a volatile gas with a mild gasoline odor. It ranks 36th in the most produced chemicals in the United States (http://www.osha.gov/SLTC/butadiene/index.html). It has an annual production of 12 billion pounds globally, with 3 billion pounds being produced in the US alone. It is produced through the processing of petroleum and is mainly used in the production of synthetic rubber, plastics and resins. Exposure to BD mainly occurs in the workplace, including the following industries: synthetic elastomer (rubber and latex) production, petroleum refining, secondary lead smelting, water treatment, agricultural

fungicides, production of raw material for nylon, and the use of fossil fuels (http://www.osha.gov/SLTC/butadiene/index.html). Exposure can also occur from automobile exhaust; polluted air and water near chemical, plastic or rubber facilities; cigarette smoke; and ingestion of foods that are contaminated from plastic or rubber containers (http://www.osha.gov/SLTC/butadiene/index.html).

Metabolism of butadiene

BD is oxidized by cytochrome P-450 2E1 and 2A6 to 1,2-epoxy-3-butene (EB) (Csanady et al., 1992; Duescher and Elfarra, 1994). EB may be further oxidized by either cytochrome P-450 2E1 or 3A4 to form 1,2:3,4-diepoxybutane (DEB) (Csanady et al., 1992; Himmelstein et al., 1995; Himmelstein et al., 1994; Malvoisin et al., 1979; Malvoisin and Roberfroid, 1982; Seaton et al., 1995). EB can be hydrolyzed by epoxide hydrolase (EH) to form 3-butene-1,2-diol (Cheng and Ruth, 1993; Malvoisin and Roberfroid, 1982; Nauhaus et al., 1996) that is metabolized by cytochrome P-450 to form hydroxymethylvinyl ketone (HMVK) (Kemper et al., 1998). Either 3-butene-1,2-diol or DEB undergo cytochrome-mediated oxidation and hydrolysis, respectively to form 3,4-epoxy-1,2-butanediol (EBD) (Boogaard and Bond, 1996; Cheng and Ruth, 1993; Malvoisin and Roberfroid, 1982). Detoxification of EB is mediated through two pathways: either by 1) direct conjugation with GSH forming monohydroxy urinary metabolites (M2) or 2) hydrolysis to 3-butene-1, 2-diol by EH that can be either directly eliminated in the urine or is conjugated with GSH, forming dihydroxy urinary metabolites (M1) (Csanady et al., 1992; Seaton et al., 1995). Fig. 1.2 shows the metabolic pathway of BD.

The electrophiles of BD metabolism include EB, DEB, EBD and HMVK that can react with nucleophilic sites in DNA to form their corresponding adducts (Cochrane and Skopek, 1994a; Cochrane and Skopek, 1994b; Powley et al., 2003). DEB is more genotoxic than EB, which in turn is more genotoxic than EBD (Adler et al., 1997; Bernardini et al., 1996; Cochrane and Skopek, 1994a). DEB is the most gentoxic due to its ability to form DNA-DNA crosslinks (Lawley and Brookes, 1967; Park et al., 2004; Park and Tretyakova, 2004). The ability of EB, DEB and EBD to induce toxicity is also affected by polymorphisms in the enzymes that metabolize them, as seen in mice where knockout of functional EH led to enhanced genotoxicity due to BD (Wickliffe et al., 2003). This is also seen in humans with polymorphic variants of the EH gene (Abdel-Rahman et al., 2001; Abdel-Rahman et al., 2003).

BD-mediated carcinogenesis is dependent on the rates of BD metabolism, the accumulation of specific oxidative metabolites in the blood or tissues and the genotoxic potential of those metabolites. The concentration of EB, DEB and EBD in blood or tissues of workers exposed to BD has not been reported in any of the studies published to date. Thus the exact metabolic fate of BD in humans is still not clear. Therefore, at this stage, we can only speculate on the probable predominant BD metabolite that is encountered in humans who are exposed to BD.

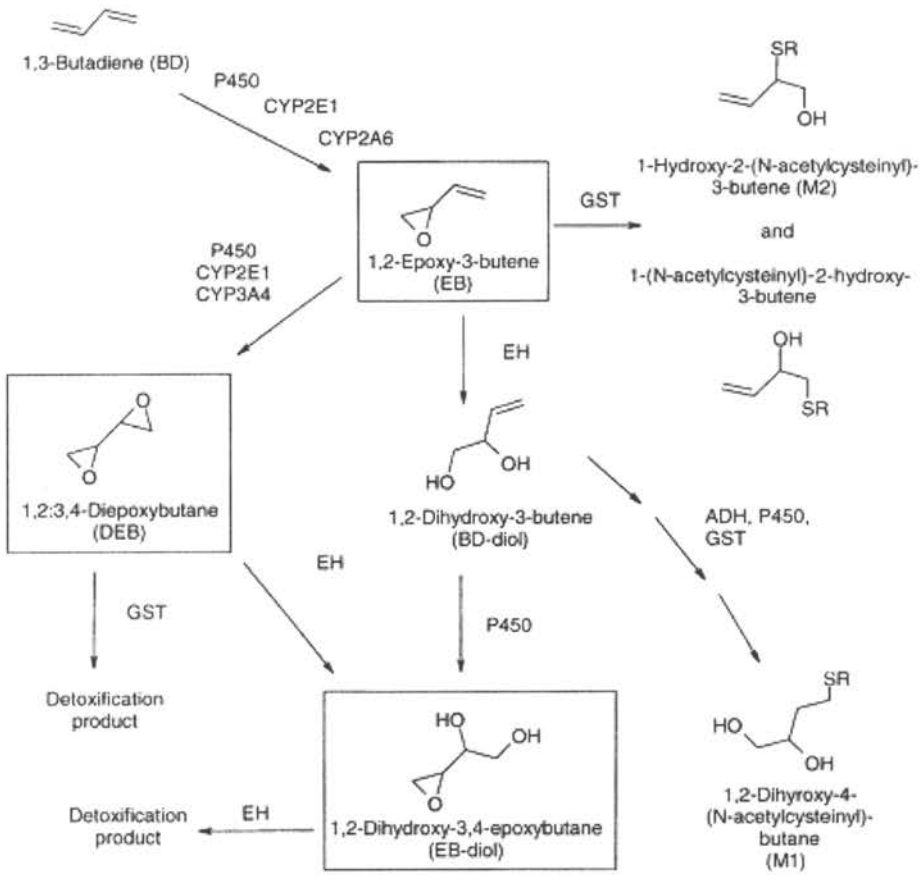


Fig 1.2. Metabolic Pathway of Butadiene. Taken from (Albertini et al., 2003)

Epidemiology

Epidemiological studies have been reported for workers exposed to both monomeric butadiene as well as polymeric butadiene, such as in the styrene-butadiene rubber industry. The largest study of monomer production workers involved 2795 individuals employed for at least six months between 1952 and 1994 (Divine and Hartman, 1996; Divine et al., 1993). In addition, there were two smaller studies of monomer production workers (Cowles et al., 1994; Downs et al., 1987; Ward et al., 1996). Haematological malignancies, mainly lymphomas were observed in one of the small studies and in the earlier update of the large cohort study. However, these findings were not consistent. Overall the evidence for cancer induction in monomer production workers has been weak and without dose-related responses. Positive correlations between exposure to BD in monomer production workers and malignancies were only seen in workers employed before 1950. These findings were different for the styrene-butadiene rubber (SBR) workers. There was an increased risk of leukemia related to exposure to BD in the largest SBR industry study consisting of 15,649 men from 8 North American SBR plants (Delzell et al., 1996; Matanoski et al., 1993; Meinhardt et al., 1982; Santos-Burgoa et al., 1992). However, no increases in other lymphohematopoietic malignancies were observed. Only job categories with relatively high BD exposures showed excess leukemia deaths. In another recent study by Graff et al (2005) that followed 16,579 workers of a SBR industry from 1943-1998, a positive association was found between BD exposure and leukemia. Overall, the epidemiological studies suggest but do not prove carcinogenicity for humans. Table 1.1. lists the characteristics of some of the important epidemiological studies.

Study design	Industry	No. of workers	Study period	Reference
Cohort	SBR	2756	1943-1976	Meinhardt et al. (1982)
Cohort	BDM	2795	1943-1994	Divine et al. (1996)
Cohort	SBR	12,110	1943-1982	Matanoski et al. (1990)
Case control	SBR	59 (LHC cases) 193 (Controls)	1943-1982	Santos Burgoa et al. (1992)
Cohort	SBR	15,649	1943-1991	Delzell et al. (1996)
Cohort	SB latex	420	1947-1986	Bond et al. (1992)
Cohort	BDM	614	1948-1989	Cowles et al. (1994)
Cohort	ABS	1037	1950-1984	Downs et al. (1992)
Cohort	BDM	364	1943-1970	Ward et al. (1995)

Table 1.1. Characteristics of the Butadiene Epidemiologic Studies. Taken from (Himmelstein et al., 1997)

ABS = acrylonitrile-butadiene-styrene plastic; BDM = butadiene monomer; LHC = lymphohaematopoeitic cancer; SB latex = styrene-butadiene latex; SBR = styrene-butadiene rubber

Rodent Studies

The limited evidence for an association between butadiene exposure and leukemia in epidemiological findings, led investigators to examine the effect of BD on rodents. BD was found to be a rodent carcinogen, inducing tumors at multiple sites in both sexes in mice and to a lesser extent in rats (Melnick et al., 1990; Owen and Glaister, 1990; Owen et al., 1987). Mice developed tumors from exposure to BD concentrations that were as much as three orders of magnitude lower than those that caused cancer in rats (Melnick et al., 1990). A two-year study of chronic inhalation exposure of female and male mice to BD concentrations as low as 20 and 62.5 ppm, respectively increased the incidence of lymphomas as well as adenomas and carcinomas of the lung and liver. In both sexes, excess tumors of the heart, forestomach, Harderian gland, and mammary glands were seen with exposures to 200 ppm (Melnick et al., 1990). At the same dose or higher, Huff et al., (Huff et al., 1985) observed an increase in lymphocytic lymphomas in B6C3F1 mice. Rats appear to be less sensitive to the effects of butadiene as seen in reports of tumor incidence at concentrations as high as 8000 ppm. Pancreatic exocrine adenomas and carcinomas and interstitial cell tumors of the testis were induced in male rats, while follicular-cell adenomas and carcinomas of the thyroid and mammary gland tumors were induced in females (Owen et al., 1987). More recently, brain tumors were found to be induced in B6C3F1 mice on exposure to BD (Kim et al., 2005). The large interspecies variations in response to BD and the inconsistencies in epidemiological studies have made it difficult to assess the exposure risk to humans. Nevertheless, BD is listed a probable human carcinogen by the International Agency for Cancer Research (IARC, 1999; USEPA, 2002).

Protein adducts induced by BD.

EB reacts with the N terminal valine of haemoglobin to produce the N-(2-hydroxy-3-butenyl)valine (HBVal) haemoglobin adducts in mice and rats exposed to this chemical by inhalation (Osterman-Golkar et al., 1991; Osterman-Golkar et al., 1993). Although potentially both DEB and EBD produce the N-(2,3,4-trihydroxybutyl)valine (THBVal) hemoglobin adducts, evidence indicates that most of these protein adducts are almost entirely derived from EBD (Perez et al., 1997). The THB Val adducts were more prevalent in humans and rodents than the HBVal adducts, thus suggesting that the concentration of EB in the blood was much lesser than that of EBD (Bond and Medinsky, 2001). Unlike DNA adducts, haemoglobin adducts are not repaired and therefore serve as a specific human biomarker depending on the lifespan of the erythrocyte.

DNA adducts induced by BD.

Several purine and pyrimidine adducts of BD have been characterized *in vitro* and *in vivo* (Citti et al., 1984; Koivisto et al., 1998; Koivisto et al., 1996; Leuratti et al., 1994; Neagu et al., 1994; Selzer and Elfarra, 1997a; Selzer and Elfarra, 1999; Tretyakova et al., 1998; Tretyakova et al., 1997; Tretyakova et al., 1996). These include N7, N1, N^2 guanine adducts; N^6, N7, N1, N9 and N3 adenine adducts; N1 inosine adducts; N3 thymidine; N3 cytidine and N3 deoxyuridine adducts. BD is not limited to the formation of monoadducts. Several DNA-DNA and DNA-protein crosslinks have been isolated and characterized. This includes the interstrand N7-dG in 5′-GNC-3′ sequences (Millard and White, 1993) and the guanine-adenine DNA interstrand crosslink (N7-dG-N^6-dA) (Park et al., 2004). Most of these adducts have been detected in rodents. The only adduct to be

detected in humans to date is the N1-(2,3,4-trihydroxybutyl)adenine (N1-THBAde) adduct with its levels ranging from <0.1 to 25 adducts/10^9 nucleotides. These adduct levels appeared to correlate to BD exposures (Zhao et al., 2000). Structures of some of these adducts are as shown in Fig.1.3.

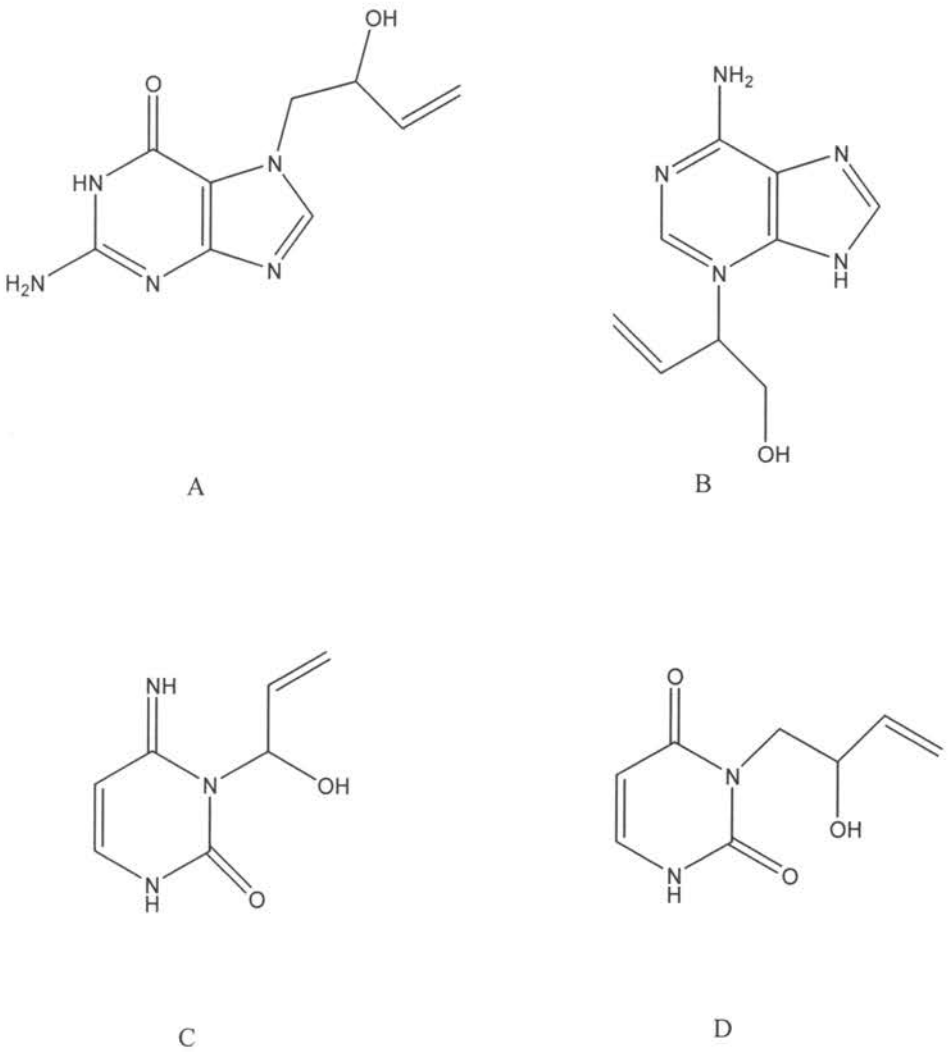


Fig 1.3. Structures of BD-derived DNA adducts. (A) N7-(2-hydroxy-3-buten-1-yl)guanine, (B) N3-(1-hydroxy-3-buten-2-yl)adenine, (C) N3-(1-hydroxy-3-buten-2-yl)cytosine, (D) N3-(2-hydroxy-3-buten-1-yl)thymine.

Methods for mutagenicity testing

A number of tests are available to determine the mutagenic potential of a chemical. These range from the traditional Ames test to the use of site-specific adducts. Some examples of these assays are as described below.

Ames test – The widely used Ames test (Ames et al., 1973) is based on the assumption that any substance that is mutagenic is also carcinogenic. This method scores for reversion mutations in a gene required for synthesis of histidine (his) in *Salmonella typhimurium*. The strain of bacteria used is unable to synthesize histidine due to a mutation in a gene required for its synthesis. Bacteria are plated on his^- plates in the presence of a test compound. The number of colonies that grow is an indication of the number of mutagenic events and a measure of mutagenicity of the compound. Therefore a reversion mutation in the *his* gene restores the bacteria's ability to synthesize histidine *de novo*. Since bacteria differ from mammals in their metabolic abilities, a drug metabolizing system from rat liver can be added to these assays. Fig. 1.4 illustrates this technique.

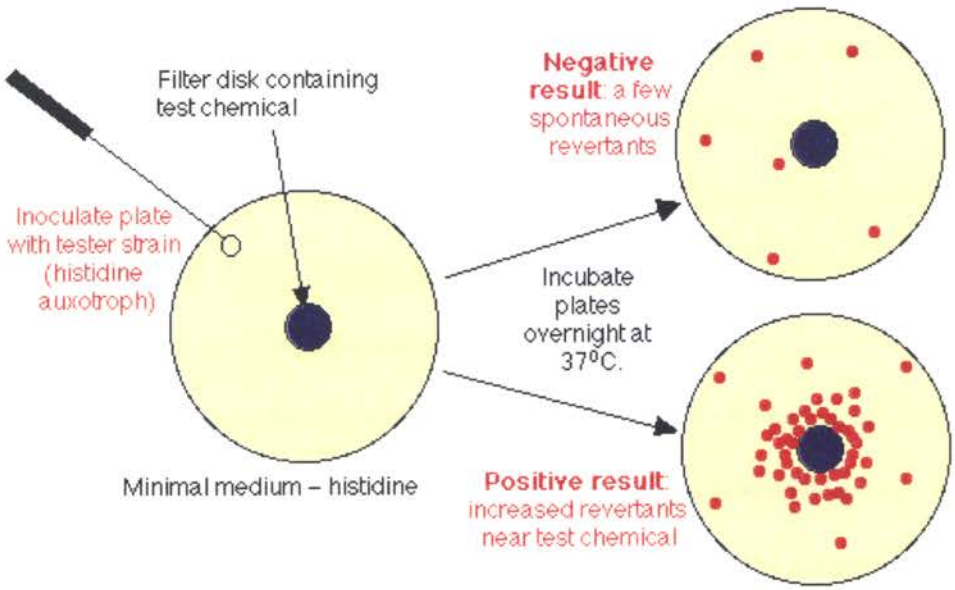


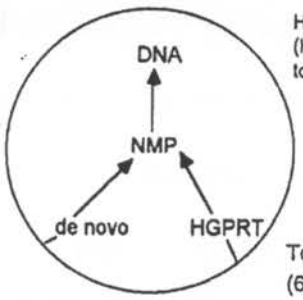
Fig.1.4. The Ames test

Mammalian mutation assay - These assays are similar to Ames test in that they are based on mutations in single copy genes. Mutations in the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) or thymidine kinase (TK) gene serve as the endpoints in this assay. HGPRT gene product is an important enzyme in the purine salvage pathway that can allow the incorporation of toxic purine analogues such as 6-thioguanine and 8-azaguanine. This leads to replication arrest and cell death. Thus wild-type cells die and the mutants survive. The TK forward assay is similar except that it uses pyrimidine analogues (Fig 1.5). The limitation of this assay like the Ames test is that positive mutagenicity does not necessarily translate to carcinogenicity of the compound (Casarett, 1996).

To provide a more realistic mutation frequency in *in vivo* assays, endogenous genes are selected for mutation detection. These include the ras and p53 genes. Following administration of a possible carcinogen, DNA is isolated from the cells and amplified. The target gene is then sequenced to obtain a mutagenic spectrum associated with that chemical. This methodology has many advantages as it takes into account the bioavailability, pharmacokinetics and DNA repair responses (Casarett, 1996).

Mammalian Cell Lines Used for Gene Mutation Studies	
Mouse lymphoma L5178Y	TK, HGPRT
CHO	HGPRT
V79 hamster cells	HGPRT

Selection of forward mutations by loss of HPGRT+ phenotype in V79 or chinese hamster ovary cells.



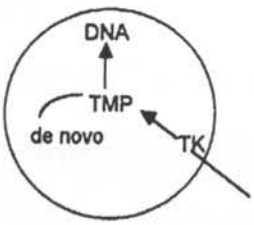
Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) is an X-linked gene that confers resistance to purine analogues such as 6-thioguanine.

Toxic Analogue (6-thioguanine)

Performed in the presence of an activation system or hepatic feeder layer

Selection of mutagen-induced TK -/- phenotype in TK+/- in mouse lymphoma assay (MOLY) in L5178Y cells. This assay detects forward mutations at the TK locus.

Treat TK (+/-) cells with test compound. Fluoruracil is converted to a toxin, killing the cells. If mutation in TK occurs, FU is not metabolized to a toxin and colonies grow.



Thymidine kinase (TK) confers resistance to pyrimidine analogues. It is a somatic gene.

Toxic analogue (bromodeoxyuridine)

Fig.1.5. HGPRT and TK mutagenicity assay. Taken from (Casarett, 1996)

The 2-year bioassay – The assay is used to determine the endpoint of tumor incidence on chronic administration of a compound to rodents. The dose given is the maximal tolerable limit (MTD) and one-half the MTD. Controls include untreated and vehicle treated rodents. The route of exposure is usually the same as the most probable route of human exposure. The B6C3F1 mouse and the F344 rat are the two strains typically used in these studies. The number of animals is set at 50 per dose per sex. Since this assay is used to extrapolate the risk from animals to humans, a number of factors need to be considered to compensate for the differences between species. The chemical should be administered in the same form as found in human exposures. The environment of the rodents and their diet should be kept constant so as to control for sources of variability among the animals (Casarett, 1996).

Genetically engineered cells and animals - The limitations associated with metabolism in various cell lines has led to the development of cell lines that have been engineered to contain various human P-450s to mimic the metabolic activation systems in humans. Transgenic mouse models have also been developed that contain bacterial based shuttle vectors with the *lacZ* or *lacI* gene. This methodology involves isolation of genomic DNA and the vector with packaging extracts and plating out with X gal and isopropylthio-β-galactoside (IPTG) on *E. coli* indicator cells. The plates are then screened for mutant colonies (white) versus wild-type colonies (blue). The DNA in the white colonies can be sequenced to determine the type of mutations induced by the specific chemical exposure (Casarett, 1996). This assay does not allow for risk estimation as its mutagenic endpoint does not prove carcinogencity of a compound.

Indirect tests for carcinogenicity detection – The indirect tests for carcinogenicity measures the induction of responses that result from DNA damage. These responses include induction of SOS repair in bacteria and unscheduled DNA synthesis in rat hepatocytes. The latter assay does not directly determine the kind of DNA damage, but the incorporation of radiolabeled thymidine during repair is used to determine the amount of damage. Sister chromatid exchange is another indirect measure of genetic damage. This assay is typically performed in CHO cells where they are treated with a dose that does not produce overt toxicity. The cells are then incubated with the thymidine analogue, bromodeoxyuridine to label actively replicated DNA. Colcemid is then added to block cells in mitosis and mitotic slides are prepared in order to examine the exchange of material among the sister chromatids during mitosis (Casarett, 1996).

^{32}P-postlabeling -^{32}P-postlabeling is a method to characterize DNA damage at the molecular level through the identification of DNA adducts.This method includes isolation of DNA that has been exposed to chemicals either *in vitro* (calf thymus DNA or nucleotides) or *in vivo* (DNA isolated from exposed rodents) followed by digestion of the DNA to individual nucleotides using micrococcal endonuclease and spleen exonuclease. Subsequently, ^{32}P is transferred to each nucleotide with T4 polynucleotide kinase. The normal nucleotides are then separated from the adducted nucleotides through thin layer chromatography (TLC) or high performance liquid chromatography (HPLC) (Casarett, 1996). Finally, nuclear magnetic resonance (NMR) is used to characterize the individual adducted nucleotides. Fig. 1.6 illustrates this methodology.

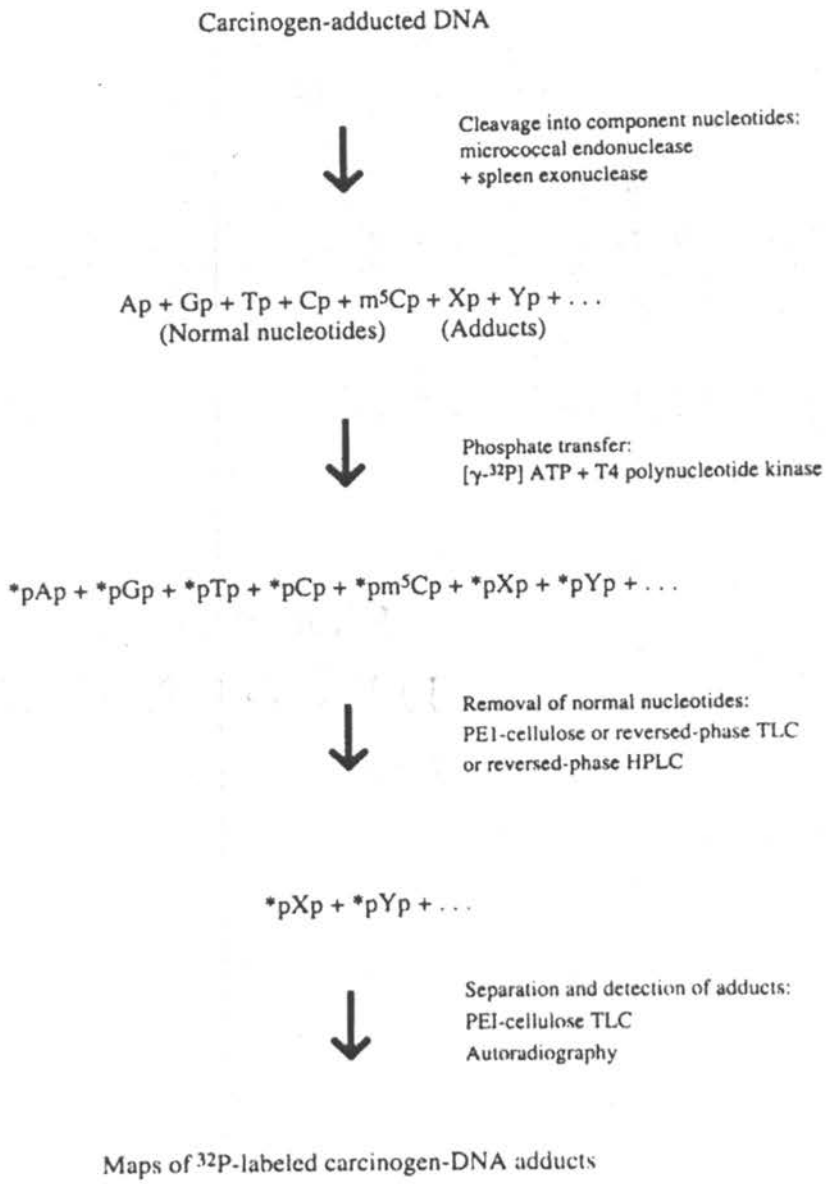


Fig 1.6. ^{32}P-Postlabeling assay for chemically adducted DNA. Taken from (Casarett, 1996)

Site-specific mutagenicity studies - Most recently, the use of methodologies to construct site-specific DNA adducts has made it possible to study the mutagenicity of individual DNA adducts. These adducts are made to replicate in either *E. coli* or mammalian systems using a phagemid or plasmid vector. The limitation of this assay is that the adduct is constructed into a specific DNA sequence. Therefore, effects of sequence context are not taken into consideration. Also, plasmid or phagemid replication is extrachromosomal and the effect of the adduct could be different within chromatin. Nevertheless, this methodology has helped to focus on which adducts contribute to a mutagenic spectrum. The site-specifically constructed BD-derived DNA adducts whose mutagenic potential has been determined include the N^2 deoxyguanosine (dG) adducts of EB and EBD (Carmical et al., 2000c), the N^6 deoxyadenosine (dA) adducts of EB and EBD (Carmical et al., 2000b), the N1-deoxyinosine (dI) adducts of EB, the N^6-N^6-dA intrastrand crosslink of DEB (Kanuri et al., 2002b) and the N^2-N^2-dG intrastrand crosslink of DEB (Carmical et al., 2000a). Table 1.2. lists the results of these site-specific mutagenicity assays.

The mutagenic potential of DNA lesions can only be directly examined by the site-specific mutagenicity method. The other methods of mutagenicity testing outlined here are only focused on the endpoints of exposure to a compound; carcinogenicity of a compound cannot be proven. Also, identification of specific kinds of DNA damage responsible for mutagenesis cannot be determined. This work utilizes the method of site specific mutagenicity to explore the mutagenic potential of the adducts described herein.

DNA Adduct	Mutagenicity in *E. coli* cells	Mutagenicity in COS-7 cells	References
N^2 dG adducts of EB and EBD	<1 %	NA	Carmical et al., 2000c
N^6 dA adducts of EB	Non-mutagenic	NA	Carmical et al., 2000b
N^6 dA adducts of EBD	<1 %	NA	
R N1-dI adduct of EB	97 %	95 %	Kanuri et al., 2002b
S N1-dI adduct of EB	53 %	59 %	
R N^6-N^6-dA intrastrand crosslink of DEB	2.8 %	19.4 %	
S N^6-N^6-dA intrastrand crosslink of DEB	8 %	54 %	
N^2-N^2 -dG intrastrand crosslink of DEB	<1% and deletions	NA	Carmical et al., 2000a

Table 1.2. Site specific mutagenicity studies of BD-derived DNA adducts

NA = not available

Polymerases

DNA adducts can also be evaluated for their mutagenic and/or bypass potential through *in vitro* studies with polymerases. Polymerases are the replication driving machinery that a cell uses to synthesize DNA. Human DNA polymerases can be classified into four different families (A, B, X and Y) based on their primary structures. Table 1.3 lists the details of the human template-dependent DNA polymerases.

Polymerase[a]	Family	Catalytic subunit				Associated activities	Proposed functions
		Molecular mass (kDa)[b]	Human gene (alias)	Chromosomal location[c]	Yeast gene[d] (alias)		
α (alpha)	B	165	*POLA*	Xp22.1-p 21.3	*POL1 (CDC17)*	Primase	chromosomal replication, S-phase checkpoint, DSB repair
β (beta)	X	39	*POLB*	8p11.2	-	dRP & AP lyase	BER, single strand break repair
γ (gamma)	A	140	*POLG*	15q25	*MIP1*	3'→5' exonuclease, dRP lyase	mitochondrial replication, mitochondrial BER
δ (delta)	B	125	*POLD1*	19q13.3	*POL3 (CDC2)*	3'→5' exonuclease	chromosomal replication, NER, BER, MMR, DSB repair
ε (epsilon)	B	255	*POLE*	12q24.3	*POL2*	3'→5' exonuclease	chromosomal replication, NER, BER, MMR, DSB repair, S-phase checkpoint
ζ (zeta)	B	353	*POLZ (REV3)*	6q21	*REV3*		TLS, DSB repair, ICL repair?, SHM
η (eta)	Y	78	*POLH (RAD30, RAD30A, XPV)*	6p21.1	*RAD30*		TLS, SHM
θ (theta)	A	198	*POLQ*	3q13.33	-		ICL repair?
ι (iota)	Y	80	*POLI (RAD30B)*	18q21.1	-	dRP lyase	TLS?, BER?, SHM
κ (kappa)	Y	76	*POLK (DINB1)*	5q13	-		TLS
λ (lambda)	X	66	*POLL*	10q23	*POL4 (POLX)*	dRP lyase	DSB repair, BER?
μ (mu)	X	55	*POLM*	7p13	-	TdT	DSB repair
σ (sigma)	X	60	*POLS (TRF4-1)*	5p15	*TRF4*		sister chromatid cohesion
REV1	Y	138	*REV1*	2q11.1-q11.2	*REV1*	TdT (for dC)	TLS

Table 1.3. Human template dependent DNA polymerases. Taken from (Shcherbakova et al., 2003) DSB-double stranded break, MMR-mismatch repair, ICL-interstrand crosslink, SHM-somatic hypermutation

E. coli DNA polymerase (pol) I was the first polymerase whose activity was identified (Kornberg et al., 1956; Lehman et al., 1958). It is a 109-kDa enzyme that has a multidomain architecture containing a polymerase activity, a 5′-3′ exonuclease activity, and a 3′-5′ exonuclease activity. The C-terminal portion of *E. coli* pol I is called the Klenow fragment (Kf) and lacks the 5′-3′ exonuclease activity (Klenow and Overgaard-Hansen, 1970). It was the first DNA polymerase structure to be solved by X-ray crystallography (Ollis et al., 1985). Structural studies of the Kf revealed that the overall shape of the polymerase domain is compared to a right hand, with subdomains referred to as *fingers, palm,* and *thumb*. Such an organization is seen for nearly all classes of polymerases solved so far. The polymerase active site, that contains catalytically essential amino acids, is contained within the palm subdomain that forms the base of the crevice surrounded by the fingers and thumb subdomains. The fingers subdomain is required for free nucleotide recognition/binding. The thumb subdomain is required for binding the DNA substrate. All DNA polymerases use a common two-metal ion mechanism to catalyze the phosphoryl transfer reaction for nucleotide addition (Steitz et al., 1994). The binding of two metal ions by the polymerase co-ordinates the interaction of the incoming nucleoside triphosphate with the 3′ OH on the primer terminus, leading to phosphodiester bond formation. Beyond these basic features, polymerases are highly diverse, between and within families. They range from relatively small proteins like the 39-kDa human DNA pol β to those as large as the 353-kDa human DNA pol ζ. A brief overview of the polymerases presented in this work is described below.

Polymerases delta and epsilon

These are the basic polymerases that carry out processive replication of chromosomal DNA during cell division in eukaryotes. It has been proposed that one of the two polymerases performs the bulk of the DNA synthesis on the leading DNA strand and the other is responsible for synthesis on the lagging strand (Burgers, 1991). Genetic studies in yeast suggest that 3'-5' exonucleases associated with the two polymerases proofread replication errors on opposite DNA strands during chromosomal (Shcherbakova and Pavlov, 1996) or plasmid (Karthikeyan et al., 2000) DNA replication. Mammalian pol δ is made up of two subunits and is structurally a heterodimer (Jiang et al., 1995; Lee et al., 1984; Lu and Byrnes, 1992; Syvaoja et al., 1990). Human pol ε consists of 2 subunits (Kesti et al., 1993). Both pol δ and pol ε replicate non-damaged DNA with high fidelity (they misincorporate at a frequency of 10^{-5}-10^{-6} on undamaged DNA) and high processivity (Shimizu et al., 2002; Thomas et al., 1991). Pol δ interacts with proliferating cell nuclear antigen (PCNA) which helps it to replicate DNA processively (Prelich et al., 1987; Tan et al., 1986). On the other hand, pol ε does not require PCNA to exhibit high processivity (Burgers, 1991). Fig. 1.7 depicts a model of the eukaryotic DNA replication fork with these polymerases.

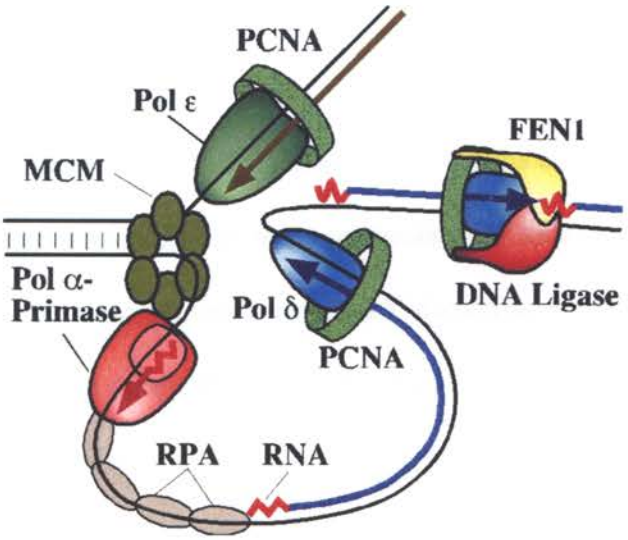


Fig. 1.7. Model of the eukaryotic DNA replication fork. In this model, PCNA is the replication processivity factor, RPA is the single-stranded DNA binding protein, FEN1 is a 5'-3'-exonuclease required for primer degradation during Okazaki fragment maturation, and MCM is the putative DNA helicase. Pol α–primase initiates leading and lagging strand synthesis. Pol ε carries out leading strand synthesis while pol δ is involved in lagging strand synthesis.

Polymerase beta

Pol β is involved in the base excision repair (BER) pathway, that repairs abasic sites (Matsumoto and Kim, 1995). The enzyme consists of an 8-kDa amino-terminal domain connected to the carboxy-terminal domain (31 kDa) by a protease-hypersensitive hinge region. The 8-kDa domain contains a 5′-deoxyribose phosphate (dRP) lyase activity that is needed during BER, whereas the large domain contains the DNA polymerase activity. The 8-kDa domain also seems to direct the polymerase to a 5′-phosphate and has single-stranded DNA binding affinity (Kumar et al., 1990). During BER, the phosphodiester backbone of an abasic site is cleaved 5′ to the sugar moiety by an abasic site endonuclease (Matsumoto and Bogenhagen, 1991). DNA pol β cleaves the remaining 5′-deoxyribose phosphate with its dRP lyase activity and fills the resulting gap using its polymerase activity (short-patch BER). The resulting nick is sealed by the action of a DNA ligase (Dianov, 2003). Alternatively, when the dRP is modified and cannot be removed by the dRP lyase activity of pol β, a DNA polymerase may incorporate 2 to 13 nucleotides through strand displacement synthesis (long-patch BER). This process generates a single-stranded flap that is cleaved by flap endonuclease 1. Pol β, in addition to Pol δ and pol ε performs DNA synthesis during long-patch BER *in vitro* (Klungland and Lindahl, 1997; Matsumoto et al., 1999; Pascucci et al., 1999; Stucki et al., 1998) and is reported to initiate the process (Podlutsky et al., 2001). Pol β does not have an associated proof-reading activity. Pol β has other roles besides BER. It has been implicated in meiotic events associated in synapsis and recombination (Plug et al., 1997) and in double stranded break repair (Wilson and Lieber, 1999). Also, mice lacking pol β

show severe growth retardation, and die immediately after birth (Sugo et al., 2000), thus indicating its importance in development.

Translesion DNA polymerases

Unlike the replicative DNA polymerases δ and ε, which authentically duplicate the genetic material, translesion DNA polymerases produce errors at a high frequency. These polymerases are specialized for replicating damaged or unusual templates that block the progression of the normal replication machinery (e.g. thymine dimers, 7, 8-dihydro-8-oxoguanine (8-oxoG) and, benzo[a]pyrene DNA adducts) due to the restrictive active sites of the replicative polymerases. There is a switching mechanism whereby, replicative polymerases get switched with translesional polymerases, allowing them to carry out bypass past the damaged DNA, followed by switching back of the replicative polymerases to resume normal replication. This will be explained in detail with pol η.

Translesional polymerases are characterized by low fidelity and processivity on undamaged DNA. They lack 3′-5′ exonuclease activity, thus making them error-prone. They possess an active site that tolerates various kinds of distortions and can accommodate bulky lesions. The four translesional polymerases presented in this work include pol η, pol ι, pol κ and pol ζ.

Polymerase eta

Human pol η is encoded by the *POLH* gene on chromosome 6 and is comprised of 713 amino acids. It is of particular interest because mutations in this gene cause the variant form of xeroderma pigmentosum (XPV) (Johnson et al., 1999a; Masutani et al.,

1999b). XP is a genetic disorder characterized by extreme sensitivity to sunlight and increased susceptibility to sunlight induced skin cancer (Lehmann, 2001). While nucleotide excision repair is deficient in XP patients, it is unaffected in XP variant patients. However, XPV cells are hypermutable to UV light (Maher et al., 1976). UV light induces two kinds of lesions cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts (6-4 PP).

Pol η can efficiently replicate past CPDs (Johnson et al., 1999a; Masutani et al., 1999b) by inserting two adenines across from the thymine dimer and it does so with the same accuracy and efficiency as opposite nondamaged DNA (Johnson et al., 2000c). However, if these CPDs are not bypassed by pol η, they are bypassed by other polymerases, which can lead to mutations resulting in skin cancer. The ability of pol η to catalyze error-free bypass of UV lesions is not restricted to the TT dimer. It is also involved in the error-free bypass of the (6-4) PPs at TC and CC sites (Yu et al., 2001). In addition to CPDs, pol η has high fidelity in replicating DNA containing other lesions. O^6-methylguanine (m^6G) is formed by the action of alkylating agents such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) on DNA. Pol η can efficiently replicate through this lesion more efficiently than pol δ, and its incorporation of the right nucleotide is twofold higher than that of pol δ (Haracska et al., 2000a). 8-oxoG is another lesion formed through oxidative damage. Pol η can also efficiently replicate past this lesion by incorporating cytosine opposite it and extending from it (Haracska et al., 2000b). Human pol η can also effectively bypass abasic sites and partially bypass (+)-*trans-anti*-benzo[a]pyrene-N^2-dG (Zhang et al., 2000a)

Pol η is specialized for lesion bypass, as its fidelity and processivity on undamaged templates is extremely low (Johnson et al., 2000c; Kusumoto et al., 2002). This suggets a switching mechanism whereby pol η is recruited to the site of damage once the replication fork is blocked by a lesion. In fact, a model is proposed to explain this process (Lehmann, 2005). Pol η colocalizes uniformly in association with replication foci during S phase (Kannouche et al., 2001). Following blockage of the replication fork, pol η accumulates at the stalled replication foci. The mammalian ortholog of Rad 18 (a DNA binding protein with ss DNA dependent ATPase activity and required for post-replication repair) binds to single-stranded DNA (Bailly et al., 1997) and together with the mammalian ortholog Rad6 (a ubiquitin conjugating enzyme that is required for post-replication repair) forms a heterodimer that monoubiquinates PCNA, a homotrimeric sliding clamp that is loaded onto the DNA at a primer terminus and is required for replication by eukaryotic polymerases (Hoege et al., 2002; Kannouche and Lehmann, 2004; Watanabe et al., 2004). This increases the affinity of PCNA for pol η. This is followed by the polymerase switch where Rad 18 and monoubiquinated PCNA recruit pol η to the site of the blocked fork (Kannouche et al., 2004; Watanabe et al., 2004). Pol η displaces pol δ. If the lesion is a TT dimer pol η performs TLS past the lesion, followed by displacement of pol η and replication by the more processive pol δ (Fig. 1.8).

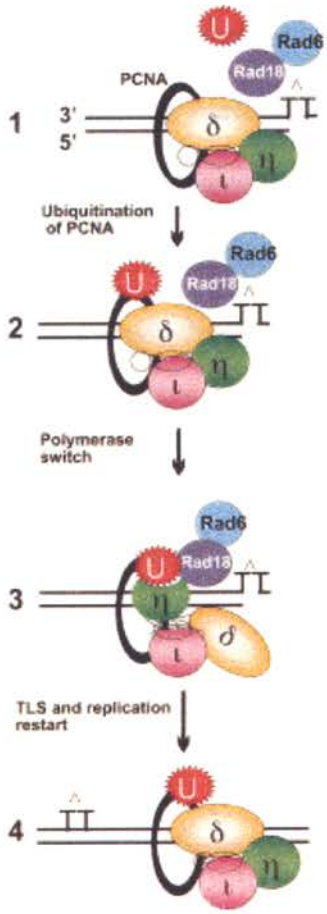


Fig 1.8. Model for translesion synthesis in mammalian cells. 1) Blockage of the fork. 2) Activation of the mammalian orthologs of Rad6-Rad-18, which monoubiquinate PCNA. 3) PCNA affinity for pol η increases and enables pol η to be switched in. 4) TLS past the damage and replication by pol δ restarts. Taken from (Lehmann, 2005).

Relative to the other eukaryotic DNA polymerases, human pol η has a low fidelity. Steady state analysis have revealed that yeast and human pol η misinsert deoxynucleotides on non-damaged DNA templates with an error rate of 10^{-2}-10^{-3} (Johnson et al., 2000c; Matsuda et al., 2000; Washington et al., 1999). The low fidelity of human pol η suggests a flexible active site, which allows the enzyme to be more tolerant of DNA distortions. This flexibility also allows human pol η to accommodate a T-T dimer in its active site despite the geometric distortions of DNA caused by the dimer (Ciarrocchi and Pedrini, 1982; Wang and Taylor, 1991), causing it to replicate past the dimer.

Polymerase iota

Pol ι is encoded by the *POLI* gene on chromosome 18 and is comprised of 715 amino acid residues (McDonald et al., 1999). In comparison to pol η and pol κ, it is a less accurate translesional polymerase. It is known for inserting nucleotides opposite various lesions. However, it is not a very good extender of template-primers (Tissier et al., 2000a). It interacts with pol η and colocalizes with pol η following UV irradiation (Kannouche et al., 2002). Pol ι also interacts with and is stimulated by PCNA (Haracska et al., 2001a). Thus it plays a role in translesion synthesis through its interaction with PCNA and pol η which helps it accumulate in the replication foci (Kannouche et al., 2003).

Pol ι shows a differential response to various DNA lesions (Zhang et al., 2001). It is blocked by 8-oxoG except at very high concentrations. Pol ι incorporates preferentially

a G opposite an abasic site and a C opposite an aminoacetofluorene-adducted dG. Under certain conditions and sequence contexts, pol ι can bypass T-T CPDs and can efficiently incorporate one or more bases opposite a T-T (6-4) PP (Vaisman et al., 2003). Pol ι can bypass a TU CPD more efficiently than a TT CPD with misincorporation of guanine in addition to adenine opposite the 3′ U of the CPD, thus decreasing the mutagenic potential of this lesion (Vaisman et al., 2006). In fact, pol ι is associated with a unique misinsertion specificity of inserting G opposite deaminated cytosines and extending from it more efficiently than correctly paired termini (Vaisman et al., 2006; Vaisman and Woodgate, 2001). In some cases like the TT (6-4) PP and abasic sites, pol ι can only serve as the incorporator and extension is carried out by another polymerase like pol ζ (Johnson et al., 2000b).

In addition to its translesion activity, pol ι also has a 5′-dRP lyase activity (Bebenek et al., 2001). It can also fill 1-3 base gaps in DNA (Bebenek et al., 2001). This suggets that pol ι may be involved in base excision repair, especially that of uracil present in DNA as pol ι is known to incorporate T opposite template A more efficiently than the other base pairs and that its gap filling ability opposite A occurs with high fidelity (Bebenek K, 2001), where it misincorporates nucleotides with frequencies of 10^{-3} to 10^{-5}.

Human DNA pol ι incorporates correct nucleotides opposite template purines with a much higher efficiency and fidelity than opposite template pyrimidines (Johnson et al., 2000b; Tissier et al., 2000b; Washington et al., 2004a). This is explained by Hoogsteen base pairing that is favored by pol ι. Crystal studies with human pol iota bound to a template G with an incoming dCTP have revealed a reduction in the C1′-C1′ distance

across the nascent base pair which in turn causes the rotation of G from *anti* to *syn* conformation and subsequent Hoogsteen base pairing with the incoming nucleotide (Fig. 1.9) (Nair et al., 2005). This is also evidenced from biochemical and mutational studies. (Johnson et al., 2005).

Pol ι accommodates an incoming base differently in its active site based on its templating sequence. When T is the template base, pol ι incorporates G with a greater efficiency than A due to a tighter binding of G to the active site, thus favoring the formation of a wobble base pair better than the correct base pair (Washington et al., 2004a). However, in comparison to the Watson-Crick base pairs, the T-G base pair is not extended efficiently by human pol ι. In fact, DNA synthesis is aborted opposite template T by Pol ι. This is known as the T stop (Zhang et al., 2000c). Another polymerase can then take on replication from the T stop.

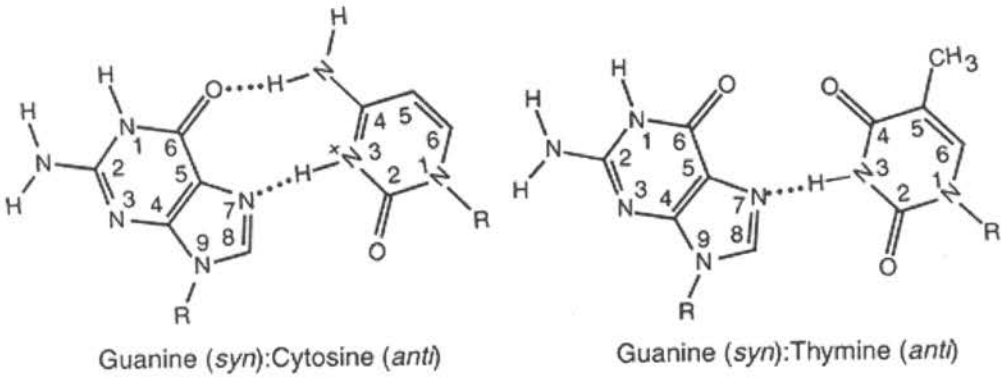


Fig 1.9. Hoogsteen base pair formation. Taken from (Prakash et al., 2005)

Polymerase kappa

Pol κ is encoded by the *POLK* gene on chromosome 5q13.1 and contains 870 amino acids. It belongs to the DinB subfamily of the Y family DNA polymerases. It misincorporates nucleotides opposite all four template bases with a frequency of ~10^{-3} to 10^{-4} (Johnson et al., 2000a). Pol κ is a well-known extender of certain lesions. It is a proficient extender of mispaired termini, extending them with a frequency of ~10^{-1} to 10^{-2} (Washington et al., 2002). It is known to induce T to G transversion mutations at a rate of 1/147 (Zhang et al., 2000b). It has moderate processivity (25 or more nucleotides), thus suggesting its role in spontaneous mutagenesis (Ohashi et al., 2000). Pol κ is also thought to be involved in untargeted mutagenesis i.e. mutagenesis induced by exogenously damaged DNA, but at sites not containing the lesion. This speculation comes from studies where untargeted mutagenesis was seen around the lesion in an aminoacetofluorene containing shuttle vector replicated in COS-7 (monkey kidney) cells (Shibutani et al., 1998) and from studies where overexpression of pol κ gene led to elevated mutagenesis (Ogi et al., 1999). Pol κ is involved in both error-free and error-prone bypass (Zhang et al., 2000b). It can bypass 8-oxoG and abasic sites incorporating an A opposite these. It can bypass aminoacetofluorene modified guanine by incorporating a C or T and less efficiently an A. It is unable to insert nucleotides opposite the 3′ T of a cis-syn TT dimer (Johnson 2000), but it can extend from a G opposite the 3′ T almost as efficiently as it can extend from an A opposite a nondamaged T (Washington et al., 2002). It is known to bypass benzo[a]pyrene guanine adducts efficiently and accurately (Zhang et al., 2002). In fact, pol κ knockout mice are sensitive to benzo[a]pyrene treatment (Ogi et al., 2002), indicating that pol κ plays a protective role through the

bypass of benzo[a]pyrene adducts. The expression of the pol κ gene is under the control of the arylhydrocarbon receptor, that is required for the activation of benzopyrene to benzopyrene diol epoxide in mammalian cells (Ogi et al., 2001). This has led to the speculation that pol κ may participate in the bypass of lesions generated by polycyclic aromatic hydrocarbons (PAHs) in a way that avoids PAH-induced mutations.

Unlike other Y family polymerases, pol κ exhibits a different localization pattern. It is found in replication foci in only a small percentage of S-phase cells. Within these foci, it does not colocalize with PCNA in the majority of cells although its interaction with PCNA stimulates its activity (Haracska et al., 2002). This reduced number of cells with pol κ foci, was seen both in untreated cells and in cells treated with hydroxyurea, UV irradiation or benzo[a]pyrene (Ogi et al., 2005).

Polymerase zeta

Pol ζ is formed from a heterodimer of Rev3 and Rev7 (Nelson et al., 1996b). It is a member of the B family of polymerases. Pol ζ is required for the majority of both spontaneous and damage-induced mutagenesis in yeast (Roche et al., 1994). It is a poorly processive enzyme, dissociating from half of template molecules after addition of 3 or more nucleotides. However in some cases, upto 200 nucleotides can be added (Nelson et al., 1996b).

Pol ζ can bypass many lesions including cis-syn TT dimer (Nelson et al., 1996b), thymine glycol (Johnson et al., 2003) and interstrand crosslinks during the G1 phase (Sarkar et al., 2006). Pol ζ is known to be an extender of mismatched termini (Johnson et al., 2000b). The efficiency of extending mispaired termini for pol ζ is ~100 fold greater

than its ability to misincorporate opposite a lesion (*reviewed in* Prakash and Prakash, 2002). It is known for acting as an extender in concert with other polymerases. For example, Rev1 can insert a C opposite an abasic site and further polymerization is carried out by pol ζ (Nelson et al., 1996a). It is inefficient at replicating through 8-oxoG and m^6G, but can readily extend from them when combined with pol δ (Haracska et al., 2003). Similarly, it can extend from a G inserted opposite the 3' T of either a T-T dimer or a (6-4) T-T PP (Johnson et al., 2001; Johnson et al., 2000b), and it efficiently extends from an A opposite an abasic site (Haracska et al., 2001b).

Pol ζ has a high fidelity in comparison to the other Y-family polymerases. It misincorporates nucleotides opposite all four template bases with a frequency of $\sim 10^{-4}$ (Haracska et al., 2003; Johnson et al., 2001; Johnson et al., 2000b). Its fidelity is more like that of pol α, that synthesizes DNA with an error rate of $\sim 10^{-4}$ (Thomas et al., 1991). It differs from other polymerases in its highly proficient ability to extend from mispaired primer termini with a frequency of 10^{-1} to 10^{-2} (Haracska et al., 2003; Johnson et al., 2001; Johnson et al., 2000b).

Besides its role in TLS, pol ζ has other functions. Disruption of the catalytic subunit of pol ζ, i.e. Rev 3 in mice leads to embryonic lethality between days 9.5 and 12.5 (Esposito et al., 2000; Wittschieben et al., 2000), suggesting that pol ζ is essential for development. Pol ζ-deficient human cells grow normally, but have reduced UV-induced mutagenesis (Zan et al., 2001). Also, loss of pol ζ causes chromosomal instability in mammalian cells (Wittschieben et al., 2006). In addition to damaged DNA, pol ζ can also take part in replication when the replication machinery is defective.

Mutations that affect replicative polymerases lead to a mutator phenotype that is dependent on functional pol ζ (Pavlov et al., 2001; Shcherbakova et al., 1996).

Given the importance of chemicals such as butadiene, the studies of its DNA adducts are warranted, especially the N3 uracil adducts which are highly stable lesions that have not yet been examined at the molecular level. In subsequent chapters, the effects of these adducts on cellular replication will be examined. Additionally *in vitro* studies determine which polymerases may be responsible for the *in vivo* bypass of these adducts. The polymerases utilized in this work include major replicative polymerases δ, and ε, repair polymerase β and translesion DNA polymerases η, ζ, κ, ι.

CHAPTER 2

SYNTHESIS AND MUTAGENESIS OF THE BUTADIENE-DERIVED N3 2′-DEOXYURIDINE ADDUCTS

ABSTRACT

The N3 2′-deoxyuridine adducts of 1,3-butadiene are a highly stable, stereoisomeric mixture of adducts derived from the reaction of cytosine with the monoepoxide metabolite of butadiene, followed by spontaneous deamination. In this study, the phosphoramidites and subsequent oligodeoxynucleotides containing the N3 2′-deoxyuridine adducts have been constructed and characterized. Using a single-stranded shuttle vector DNA, the mutagenic potential of these adducts has been tested following replication in mammalian cells. Replication past the N3 2′-deoxyuridine adducts was found to be highly mutagenic with an overall mutation yield of ~ 97%. The major mutations that were observed were C to T transitions and C to A transversions. *In vitro,* these adducts posed a complete block to both the Klenow fragment of *Escherichia coli* polymerase I and pol ε, while these lesions were significantly blocking to pol δ and pol β. These data suggested a possible involvement of bypass polymerases in the *in vivo* replication of these lesions. Overall these findings indicate that the N3 2′-deoxyuridine adducts are highly mutagenic lesions that may contribute to butadiene-mediated carcinogenesis.

INTRODUCTION

BD is a high volume, industrial chemical used in the manufacture of synthetic rubber, plastics and resins. BD is also a constituent of cigarette smoke, automobile exhaust and gasoline vapor, thus making it a relevant environmental hazard (Brunnemann et al., 1990; Pelz et al., 1990). It is an established rodent carcinogen with its effect being more pronounced in mice than rats (Melnick et al., 1990; Owen et al., 1987). BD-induced specific genetic alterations lead to the development of lymphomas, brain tumors and mammary adenocarcinomas in mice (Kim et al., 2005; Zhuang et al., 1997; Zhuang et al., 2002). Occupational exposure to BD is correlated with an increased risk of developing leukemia (Delzell et al., 1996; Graff et al., 2005; Macaluso et al., 1996; Santos-Burgoa et al., 1992). BD has been classified by the International Agency for Research on Cancer as a probable human carcinogen (IARC, 1999). More recently, it has been classified by the National Toxicology Program as a known human carcinogen (2001) and by the US Environmental Protection Agency as carcinogenic to humans by inhalation (USEPA, 2002).

The mechanisms underlying BD-induced carcinogenesis are not clearly understood. Since covalent modification of DNA represents the initial step to carcinogenesis, one may gain a better understanding of this process through the study of DNA adducts of BD. To date, many BD-derived adducts have been detected following treatment of DNA *in vitro,* as well as in DNA isolated from exposed organisms. These include N1, N^2, and N7 adducts of deoxyguanosine (Boogaard et al., 2001; Powley et al., 2005; Selzer and Elfarra, 1996b; Zhang and Elfarra, 2004), N1, N3, N^6 adducts of adenine (Selzer and Elfarra, 1996a; Selzer and Elfarra, 1999; Tretyakova et al., 1998;

Zhao et al., 2000) and the N3 adducts of cytidine and thymidine (Selzer and Elfarra, 1997a; Selzer and Elfarra, 1997b). Some of these adducts (e.g. N7 guanine and N3 adenine) are unstable, giving rise to apurinic sites. Site-specific mutagenesis studies have made it possible to examine the role of some of the other adducts. These include previous studies in our lab that have demonstrated that N^6 deoxyadenosine and N^2 deoxyguanosine adducts are weakly mutagenic in *E. coli* (Carmical et al., 2000b; Carmical et al., 2000c), while the adducts of N1 deoxyinosine and the N^6,N^6 -deoxyadenosine intrastrand cross-link were considerably mutagenic in *E. coli* and mammalian cells (Kanuri et al., 2002b).

The role of BD-derived pyrimidine adducts has not yet been examined, particularly the N3 cytosine adducts formed from the reaction of EB with deoxycytidine or calf thymus DNA (Selzer and Elfarra, 1997b; Selzer and Elfarra, 1999). These adducts were unstable due to epoxidation and they subsequently deaminated to form the corresponding BD N3-dU adducts. These BD N3-dU adducts were stable towards hydrolysis in DNA and as such, represent a potential source of mutagenesis, since this type of alkylation not only changes the base pairing of the parent nucleoside, but also has the potential to block DNA replication enzymes. Mutations at GC base pairs are critical events in BD mutagenicity as indicated by studies of mutations in *lacI* and *lacZ* mice and *hprt* mutational analyses in rodents and humans *(reviewed in* Jackson et al., 2000), thus suggesting modification of cytosine by BD metabolites and formation of the N3-dU adducts *in vivo*.

Therefore, the current study focuses on the synthesis of these BD N3-dU adducts, their incorporation into oligodeoxynucleotides and their subsequent use in cellular and *in vitro* studies to examine their effects on replication.

MATERIALS AND METHODS

Materials. All solvents and reagents for the synthesis of the BD N3-dU were obtained from Fisher-Acros or Aldrich Chemicals except for bis-N,N,N′,N′-tetraisopropyl–cyanoethyl phosphine which was obtained from Chemgenes (Cambridge, MA). MALDI-TOF (matrix assisted laser desorption/ionization time of flight) mass spectra of modified oligodeoxynucleotides were performed on an ABI Voyager 4211 system using α-cyano-4-hydroxycinnamic acid as the matrix and calibrated against 10 kDa and 4 kDa oligodeoxynucleotide standards from Genosys. T4 DNA ligase, T4 polynucleotide kinase, *Eco*RV, and Klenow Fragment (Kf) of *E. coli* polymerase I and Kf exo$^-$ were obtained from New England BioLabs (Ipswich, MA). S1 nuclease and proteinase K were purchased from Invitrogen (Rockville, MD). Calf thymus DNA pol δ and PCNA were generous gifts from Dr. K. M. Downey (University of Miami, Miami, FL). Pol ε from HeLa cells was kindly provided by Dr. S. Linn (University of California, Berkeley, CA) and yeast DNA pol δ was a generous gift from Dr. P.M. Burgers (Washington University School of Medicine, St. Louis, MO). Human pol β was obtained from Enzymax (Lexington, KY). ss pMS2 DNA was a generous gift from Dr. M. Moriya (State University of New York, Stony Brook, NY). [γ^{32}P] Ribo-ATP was purchased from PerkinElmer Life Science (Boston, MA). Bio-Spin columns were purchased from Bio-Rad (Hercules, CA). Centricon 100 concentrators were obtained from Amicon Inc. (Beverly, MA). Dulbecco's modified Eagle's medium, fetal bovine serum, Opti-MEM (reduced serum medium), L-glutamine, antibiotic-antimycotic, and Lipofectin reagent for tissue culture were obtained from Invitrogen (Rockville, MD). Trypsin-EDTA and HEPES buffer were purchased from Cellgro Mediatech (Herndon, VA). COS-7 cells were

purchased from American Type Culture Collection (Rockville, MD). Nondamaged 12-mer and 32-mer with a deoxycytosine (dC) in place of BD N3-dU, were purchased from Midland Reagent Co. (Midland, TX).

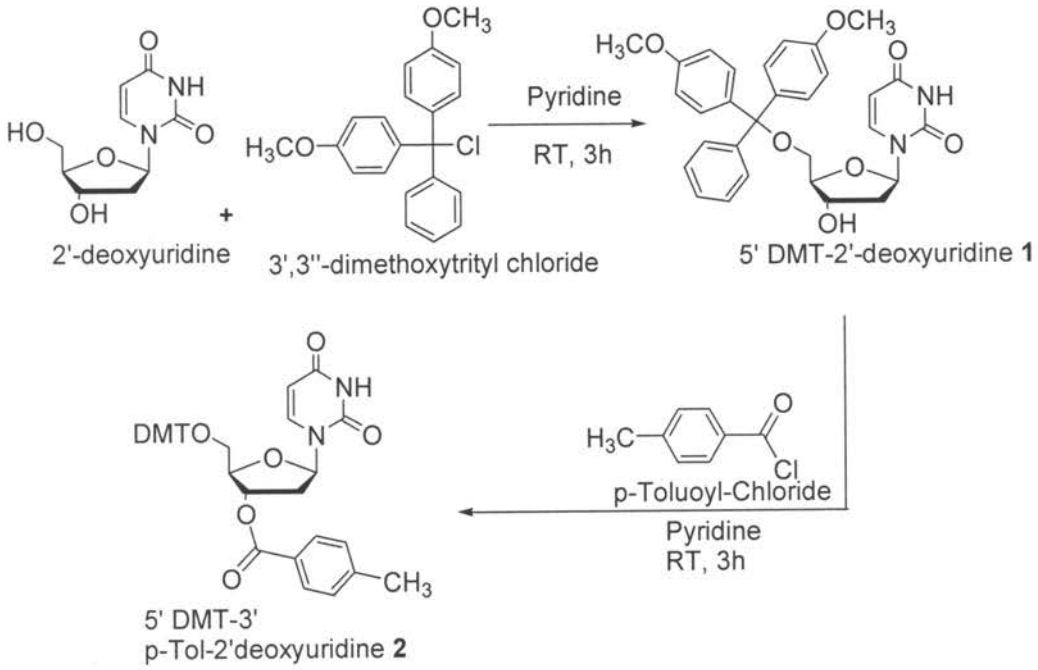
Synthesis of the BD N3-dU adducts and their incorporation into oligodeoxynucleotides.

The following work was carried out by Linda Hackfeld and Dr. Richard Hodge at UTMB, Galveston, TX.

a) Synthesis of 5′-O-Dimethoxytrityl-2′-deoxyuridine (DMT-dU).

Synthesis of DMT-dU was performed based on methods described by Beaucage et al. (Beaucage, 1992). 2′-dU (5 g, 21.9 mmol) was co-evaporated twice with anhydrous pyridine in a 250 ml flask, and re-dissolved with 100 ml fresh anhydrous pyridine under argon. Dimethoxytrityl chloride (DMT-Cl) (4.45 g, 13.14 mmol) was added at 0°C (ice bath) and stirred under argon for 30 min. A second addition of DMT-Cl (4.45 g, 13.14 mmol) was then introduced at 0°C and after 10 min the ice bath was removed and the reaction was stirred for 2 h. The reaction was monitored by thin layer chromatography (TLC) using 100% ethyl acetate as eluant (starting material relative front (Rf) = 0.01, product Rf = 0.40). The reaction was quenched by cooling to 0°C, followed by addition of 40 ml methanol and stirring for 15 min. The reaction mixture was then evaporated to a minimum under reduced pressure, and subsequently redissolved in 100 ml methylene chloride. The mixture was then neutralized with 5% aqueous sodium bicarbonate while stirring until the aqueous layer remained at pH 8. The layers were then separated and the aqueous layer extracted a second time with fresh methylene chloride. The organic layers were then combined, dried over sodium sulfate, filtered and evaporated to give 14.2 g

crude DMT-dU. This was purified by silica gel chromatography using a step gradient from 49.9% ethyl acetate : 49.9% hexane : 0.2% triethylamine to 99.8% ethyl acetate : 0.2% triethylamine. Fractions containing the product were combined and evaporated to give 10.2 g (87.7%) DMT-dU or product **1** (Scheme 2.1). ^{1}H NMR (d_6-DMSO): δ 11.3 (s, 1H, NH), 7.63 (d, 1H, J = 7.53, H-6), 7.37 (d, 2H, J = 7.87, DMT), 7.31 (m, 2H, DMT), 7.24 (m, 5H, DMT), 6.90 (m, 4H, DMT), 6.14 (m, 1H, H-1′), 5.37 (d, 1H, J = 7.87, H-5), 5.32 (d, 1H, J = 4.79, 3′-OH), 4.27 (m, 1H, H-4′), 3.87 (m, 1H, H-3′), 3.74 (s, 6H, DMT-OCH_3s), 3.23 (m, 1H, H-5′), 3.17 (m, 1H, H-5″), 2.18 (m, 2H, H-2′, H-2″).



Scheme 2.1. Synthesis of 5′-DMT-3′p-Tol-2′deoxyuridine.

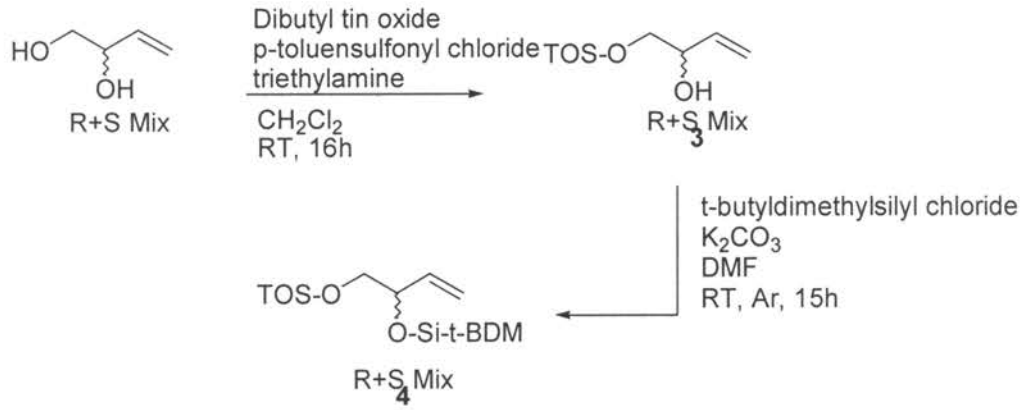
b) Synthesis of 5′-O-DMT-3′-O-toluoyl-2′-deoxyuridine (DMT-dU-Tol).

DMT-dU (500 mg, 0.942 mmol) was added to a 100 ml flask with 5 ml dry pyridine under argon with stirring. Once dissolved, 150 µl of p-toluoyl chloride (1.13 mmol) was added and the mixture stirred for 3 h at room temperature (RT) under argon. The reaction was monitored by TLC using 100% ethyl acetate as the eluant (starting material Rf = 0.40, product Rf = 0.75). The product was recovered by diluting the mixture with 20 ml methylene chloride and carefully washing with 20 ml saturated sodium bicarbonate. The organic layer was then separated, dried over sodium sulfate, filtered and evaporated to yield 720 mg crude product that was then purified by silica gel chromatography using 49.9% ethyl acetate: 49.9% hexane: 0.2% triethylamine as eluant. The pure product fractions were pooled and evaporated to yield 460 mg (75%) of product **2** (Scheme 2.1). ^{1}H NMR (d_6-DMSO): δ 11.3 (br s, 1H, N3-H), 7.89 (d, 2H, J = 6.84, p-toluoyl), 7.69 (d, 1H, J = 7.87, H-6), 7.36 (m, 4H, DMT + p-toluoyl), 7.29 (m, 2H, DMT), 7.24 (m, 5H, DMT), 6.87 (m, 4H, DMT), 6.23 (m, 1H, H-1′), 5.50 (m, 1H, H-3′), 5.47 (d, 1H, J = 7.87, H-5), 4.23 (m, 1H, H-4′), 3.72 (s, 6H, DMT-OCH_3s), 3.41 (m, 2H, H-5′, H-5″), 2.59 (m, 2H, H-2′, H-2″), 2.40 (s, 3Hs, p-toluoyl CH_3s).

c) Synthesis of 2-hydroxy-3-butene-1-(p-toluenesulfonate).

This synthesis was based on methods used by Martinelli et al (Martinelli et al., 2002). Dibutyltin oxide (2.82 g, 11.35 mmol) was added to 1,2-dihydroxy-3-butene (R,S mixture) (10 g, 113.5 mmol) in methylene chloride (200 ml). Toluenesulfonyl chloride (21.6 g, 113.5 mmol) and triethylamine (16 ml, 113.5 mmol) were added and the mixture stirred at RT overnight. Water (50 ml) was added and the layers separated. The aqueous layer was extracted twice with methylene chloride (50 ml each) and the combined

organic layers were washed with water and saturated NaCl solution (100 ml each), dried over sodium sulfate, filtered and evaporated to give **3** (Scheme 2.2) as an oil that crystallized upon cooling (26.8 g, 97% yield). ^{1}H NMR ($CDCl_3$) δ 7.78 (d, J = 8.27, 2H, Tos-ortho Hs), 7.33 (d, J = 8.07, 2H, Tos-meta Hs), 5.73 (m, 1H, H-3), 5.34 (d, J = 17.15, 1H, H-4a), 5.21 (d, J = 10.49, 1H, H-4b), 4.37 (m, 1H, H-2), 4.03 (dd, J = 3.43, 1H, H-1b), 3.89 (dd, J = 7.26, 1H, H-a), 2.50 (s, 1H, OH), 2.41 (s, 3H, Tos-CH_3).



Scheme 2.2. Synthesis of 2-O-t-butyldimethylsilyl-3-butene-1-(p-toluenesulfonate).

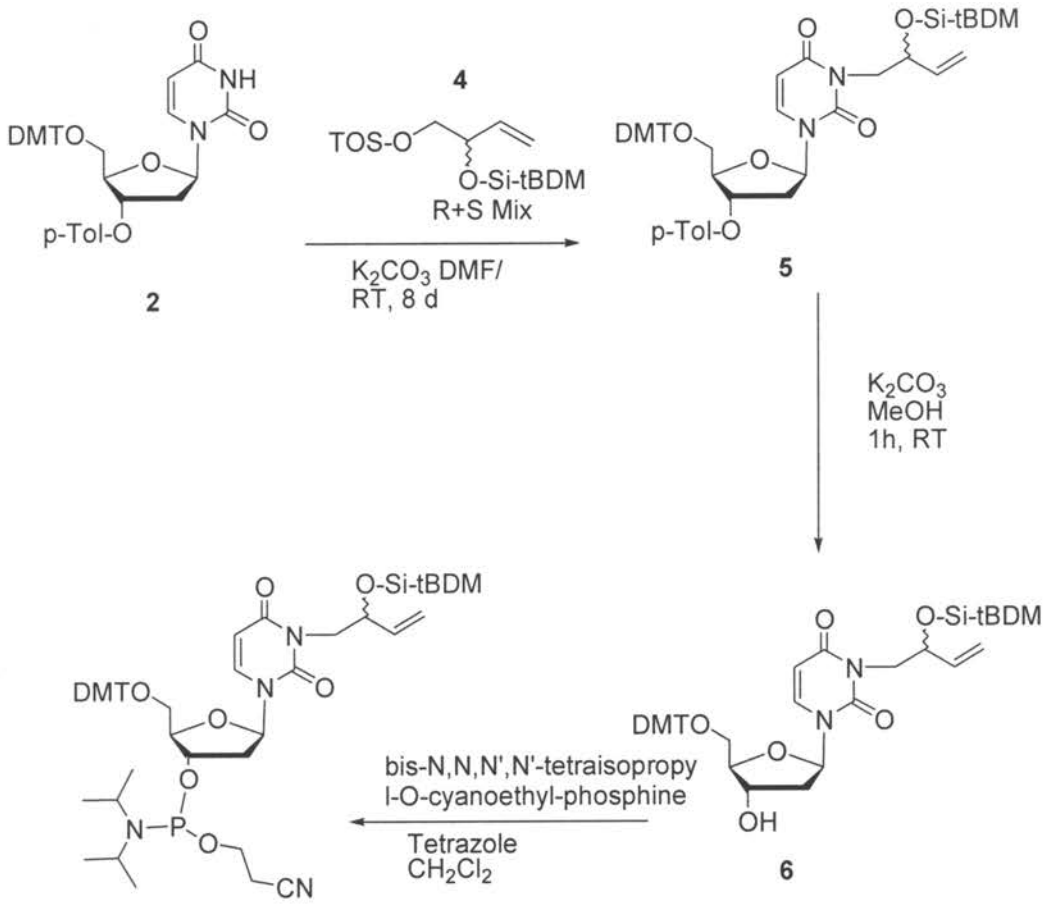
d) Synthesis of 2-O-t-butyldimethylsilyl-3-butene-1-(p-toluenesulfonate).

2-Hydroxy-3-butene-1-(p-toluenesulfonate) (130 mg, 0.537 mmol) was added to 500 µl anhydrous dimethylformamide under argon. Anhydrous potassium carbonate (74.2 mg, 0.537 mmol) was added and the mixture stirred under argon for 5 min. Tert-butyldimethylsilyl chloride (89 mg, 0.59 mmol) was then added and the mixture stirred under argon at RT overnight. The reaction was assayed by TLC (20% ethyl acetate/hexane). The reaction products showed over an 80% yield (Rf = 0.85). The mixture was then filtered of solids, washed with methylene chloride, evaporated to a minimum and purified by flash chromatography column (10-20% gradient ethyl acetate/hexane) to give **4** (86.7 mg, 45.7%) along with 25 mg recovered **3** (19.2%) (Scheme 2.2). ^{1}H NMR ($CDCl_3$) δ 7.78 (d, 2H, J = 7.76, Tos-ortho Hs), 7.34 (d, 2H, J = 7.76, Tos-meta Hs), 5.71 (m, 1H, H-3), 5.31 (d, 1H, J = 17.22, H-4 trans), 5.17 (d, 1H, J = 10.13, H-4 cis), 4.34 (m, 1H, H-2), 3.91 (m, 1H, H-1a), 3.82 (m, 1H, H-1b), 2.45 (s, 3H, Tos-CH_3), 0.86 (s, 9H, t-Butyl-Si-CH_3s), 0.04 (s, 3H, Si-CH_3), 0.03 (s, 3H, Si-CH_3).

e) Synthesis of 5′-O-DMT-3′-O-toluoyl -N3-(2-O-tert-butyldimethylsilyl (tBDMSi)-3-butene) -2′-deoxyuridine, (DMT-dU-(Bu-OSi)-Tol).

DMT-dU-Tol (350 mg, 0.54 mmol) was dissolved in 5 ml anhydrous dimethylsulfoxide in a 20 ml flask under argon with stirring. Anhydrous potassium carbonate (100 mg) was added and the mixture stirred under argon for 10 min before adding 2-O-t-butyldimethylsilyl-3-butene-1-(p-toluenesulfonate) (200 mg, 0.562 mmol). The mixture was sealed and stirred under argon for 8 days. The crude mixture was added directly to a silica gel column packed in 25% ethyl acetate/hexane and eluted by step gradient to 33%

ethyl acetate/hexane resulting in a yield of 215 mg of product **5** (47%) (Scheme 2.3). ^{1}H NMR ($CDCl_3$) δ 7.94 (d, 2H, J = 8.06, p-toluoyl-ortho Hs), 7.69 (m, 1H, H-6), 7.30 (d, 2H, J = 8.06, p-toluoyl-meta Hs), 7.27 (m, 5H, DMT-Bz), 7.17 (d, 4H, J = 8.79, DMT-ortho Hs), 6.84 (d, 4H, J = 8.42, DMT-meta Hs), 6.34 (m, 1H, H-1′), 5.86 (m, 1H, butenol H-3), 5.81 (d, 1H, J = 8.06, H-5), 5.57 (m, 1H, H-3′), 5.24 (d, 1H, J = 17.58, butenol H-4 trans), 5.12 (d, 1H, J = 9.16, butenol H-4 cis), 4.55 (m, 1H, butenol H-2), 4.26 (s, 1H, H-4′), 4.17 (m, 1H, butenol H-1a), 4.01 (s, 2H, H-5′, 5″), 3.83 (m, 1H, butenol H-1b), 3.81 (s, 3H, DMT-OCH_3), 3.80 (s, 3H, DMT-OCH_3), 2.59 (m, 1H, H-2′), 2.50 (m, 1H, H-2″), 2.44 (s, 3H, p-toluoyl-CH_3), 0.85 (s, 9H, silyl t-butyl CH_3s), -0.01 (s, 3H, silyl CH_3), -0.04 (s, 3H, silyl CH_3).



5'-DMT-N3-(3-buten-2-O-t-butyldimethylsilyl-1 yl)-2' deoxyuridine-3'-O-(N,N,-diisopropyl-O-cyanoe thyl)- phosphoramidite **7**

Scheme 2.3. Synthesis of 5′-DMT-N3-(3-buten-2-O-t-butyldimethylsilyl-1yl) -2′ deoxyuridine-3′-O-(N, N-diisopropyl-O-cyanoethyl)-phosphoramidite.

f) Synthesis of 5′-O-DMT-2′-deoxyuridine-N3-(2-O-tBDMSi-3-butene), (DMT-dU-Bu-OSi).

DMT-dU-(Bu-OSi)-Tol (190 mg, 0.228 mmol) was dissolved in 10 ml anhydrous methanol in a sealed 50 ml flask under argon. To this was added 276 mg anhydrous potassium carbonate and the suspension stirred under argon for 3 h. TLC (49.9% ethyl acetate: 49.9% hexane: 0.2% triethylamine) showed complete deprotection. The reaction was quenched with 40 ml 0.5 M sodium phosphate buffer (pH 5.5) and quickly extracted with 100 ml methylene chloride. The methylene chloride fraction was dried over sodium sulfate, filtered and evaporated. The crude product was purified by preparative CycloGraph TLC using a 25% ethyl acetate to 50% ethyl acetate (in 1% ethylamine/hexane) step gradient. The product was monitored by UV 254 nm and collected to yield 160 mg of **6** (98%) (Scheme 2.3). HPLC analysis showed the product to be greater than 99% pure. ^{1}H NMR ($CDCl_3$) δ 7.53 (m, 1H, H-6), 7.27 (m, 5H, DMT-Bz), 7.18 (d, 4H, J = 7.69, DMT- ortho Hs), 6.83 (d, 4H, J = 7.69, DMT-meta Hs), 6.17 (m, 1H, H-1′), 5.85 (m, 1H, butenol H-3), 5.77 (d, 1H, J = 7.69, H-5), 5.23 (d, 1H, J = 17.21, butenol H-4 trans), 5.11 (d, 1H, J = 10.26, butenol H-4 cis), 4.59 (m, 1H, H-3′), 4.53 (m, 1H, butenol H-2), 4.15 (m, 1H, butenol H-1a), 4.03 (s, 1H, H-4′), 3.94 (m, 1H, butenol H-1b), 3.84 (m, 2H, H-5′, 5″), 3.81 (s, 6H, DMT-OCH_3s), 2.40 (m, 2H, H-2′, 2″), 0.84 (s, 9H, silyl t-butyl CH_3s), -0.01 (s, 3H, silyl CH_3), -0.06 (s, 3H, silyl CH_3).

g) Synthesis of 5′-O-DMT-2′-deoxyuridine-N3-(2-O-tBDMSi-3-butene)-3′-O-(O-cyanoethyl- N,N-diisopropyl), (DMT-dU-BuOSi-CE) phosphoramidite.

DMT-dU-Bu-OSi (112 mg, 0.16 mmol) **6** was dissolved with 1 ml dry methylene chloride in a 20 ml flask under argon. To this was added simultaneously via syringe, while stirring under argon, 71 µl (0.224 mmol) neat bis-N,N,N′,N′-tetra-isopropyl-O-cyanoethyl-phosphine and 448 µl (0.192 mmol) tetrazole solution in tetrahydrofuran (30 ug/µl). The solution was stirred for 1 h under argon at RT. TLC (29.9% ethyl acetate: 69.9% hexane: 0.2% triethylamine, pre-run TLC plates) showed that the reaction had proceeded to completion. The mixture was extracted with ethyl acetate and 5% sodium bicarbonate, and the ethyl acetate fraction further extracted with saturated aqueous NaCl. The ethyl acetate fraction was then dried over sodium sulfate, filtered and evaporated. The crude product was purified using a preparative TLC CycloGraph system pre-wetted and run with the same TLC solvent above. Pure product fractions were pooled to yield 95 mg pure product **7** (67%) (Scheme 2.3). ^{1}H NMR (300MHz, CD_3CN) δ 7.63 (m, 1H, H-6), 7.42 (m, 2H, DMT-ortho Hs), 7.25 (m, 7H, DMT- ortho Hs and Bz), 6.85 (m, 4H, DMT-meta Hs), 6.19 (m, 1H, H-1′), 5.84 (m, 1H, butenol H-3), 5.41 (m, 1H, H-5), 5.23 (d, 1H, J = 17.3, butenol H-4 trans), 5.10 (d, 1H, *J* = 10.5, butenol H-4 cis), 4.60 (m, 1H, H-3′), 4.53 (m, 1H, butenol H-2), 4.05 (m, 2H, butenol H-1a and H-4′), 3.76, (s, 3H, DMT-OCH_3), 3.75 (s, 3H, DMT-OCH_3), 3.62 (m, 1H, butenol H-1b), 3.60 (m, 2H, H-5′, 5″), 3.33 (m, 2H, isopropyl CH_3s) 2.63 (t, 1H, OCH, cyanoethyl), 2.52 (t, 1H, OCH, cyanoethyl), 2.42 (m, 1H, H-2′), 2.27 (m, 1H, H-2″), 1.93 (m, 2H, CH_2CN), 1.16 (m, 6H, isopropyl CH_3s), 0.82 (s, 9H, silyl t-butyl CH_3s), -0.03 (s, 3H, silyl CH_3), -0.10 (s, 3H, silyl CH_3). ^{31}P NMR (121.5 MHz, CD_3CN) δ 147.3, 147.2.

h) Oligodeoxynucleotide Synthesis and Purification.

Two oligodeoxynucleotides (12-mer 5′-GCTAGC**X**AGTCC-3′ and 32-mer 5′-ACCATGCCTGCAAGAA**X**TAAGCAATGATCGCC-3′) were synthesized incorporating N3-(3-butene-2-O-t-butyldimethylsilyl)-2′-deoxyuridine in place of a 2′-deoxycytidine at site **X** using phosphoramidite chemistry and standard oligodeoxynucleotide synthesis procedures. Oligodeoxynucleotides were synthesized at the 1.0 μM scale using a PerSeptive Biosystems Expedite 8909 DNA synthesizer with off-line coupling of the DMT-dU-BuOSi-CE phosphoramidite by placing a hold in the sequence program at the coupling step and introducing the modified nucleoside by mixing of the phosphoramidite (10 mg/100 μl anhydrous acetonitrile) and tetrazole (3 mg/100 μl anhydrous acetonitrile) solutions across the synthesis cassette via syringes. The coupling was carried out for 20 min with occasional mixing, followed by placement of the cassette back on the synthesizer, washing with acetonitrile and continuation of the sequence synthesis program. Standard phosphoramidites were used for all other coupled bases and the final 5′-dimethoxytrityl group was left on. Oligodeoxynucleotide cassettes were treated using standard cleavage from the support (1 h concentrated ammonia at RT) and deprotection of the resulting oligodeoxynucleotide/ammonia solution (60°C for 16 h). Following deprotection, an aliquot of the crude sample was subjected to analytical HPLC analysis (Waters Delta Pak C-18, 0-20% acetonitrile gradient in 0.1 M ammonium formate (pH 7.2) for 20 min, at 1 ml/min flow rate) and the bulk of the product was evaporated to a minimum on a Savant SpeedVac system. The 5′-dimethoxytrityl containing full-length oligodeoxynucleotides were then purified on C-18 Sep Pak cartridges. A sample of this material was also analyzed by HPLC using the same system with a shallower gradient (4-10% acetonitrile, 20 min). Purification of the

base-protected oligodeoxynucleotides after DMT removal was conducted on a preparative Hamilton PRP column using a 5 ml/min flow rate and gradient of 2-10% acetonitrile in 0.1 M ammonium formate (pH 7.2).

i) Deprotection of t-BDMSi from the oligodeoxynucleotides.

The remaining t-butyldimethylsilyl protecting groups on the butenol side chain on N3 adducted deoxyuridine oligodeoxynucleotides were removed using 200 µl of a 67/33 mixture of anhydrous 1.0 M tetrabutylammonium fluoride/neat triethylammonium hydrofluoride solution overnight at 0-4°C, followed by Nap-10 purification against 0.1 M sodium phosphate buffer (pH 7.2) to remove residual tert-butyldimethylsilyl fluoride and reagents. This was necessary since triethylammonium hydrofluoride alone was too acidic, causing noticeable degradation of the oligodeoxynucleotides, whereas tetrabutylammonium fluoride deprotection alone failed to achieve greater than 60% deprotection. All products were analyzed by HPLC and MALDI-TOF mass spectrometry. The observed pre and post desilylation mass spectrometric values (versus calculated values) were 3789.4 (3789.7) and 3675.4 (3675.7) for the 12-mer and 9951.6 (9951.8) and 9836.5 (9837.7) and for the 32-mer, respectively, within 0.012% of instrument accuracy.

This completes the work and analyses performed by Linda Hackfeld and Richard Hodge.

Characterization of the 12-mer oligodeoxynucleotide containing the BD N3-dU adduct.

The following work was carried out by Dr. Ivan Kozekov at Vanderbilt University, Tennessee

a) Capillary Gel Electrophoresis (CGE).

Electrophoretic analyses were carried out using a Beckman P/ACE MDQ instrument system (using 32 Karat software, version 5.0) monitored at 260 nm on a 31.2 cm x 100 μm eCAP capillary, with samples applied at 10 kV and run at 9 kV. The capillary was packed with 100-R gel (for single-stranded DNA) using the Tris-borate buffer system containing 7 M urea.

b) Enzymatic Hydrolysis.

The 12 mer oligodeoxynucleotide 5′-GCTAGC**X**AGTCC-3′ (0.3 A_{260} units) was dissolved in 30 μl of 10 mM Tris-HCl buffer (pH 7.0) containing 10 mM $MgCl_2$ and incubated with DNase I (8 units, Promega), snake venom phosphodiesterase I (0.02 units, Sigma), and alkaline phosphatase (1.7 units, *E. coli*; Sigma) at 37°C for 24 h. The mixture was analyzed by reversed phase HPLC. The adducted nucleosides were identified by comparison with authentic samples based on retention times, co-injection, and ultraviolet spectra. HPLC analyses of the enzymatic digestion were performed on a Beckman HPLC system (32 Karat software version 3.1, pump module 125) with a diode array UV detector (module 168) monitoring at 260 nm using a Waters YMC ODS-AQ columns (250 mm x 4.6 mm i.d., 1.5 ml/min) with H_2O and CH_3CN using the following gradient: 1-10% acetonitrile over 15 min, 10-20% acetonitrile over 5 min, hold for 5 min, 100% acetonitrile over 3 min, hold for 2 min, and then to 1% acetonitrile over 3 min.

c) DNA Sequencing.

The 12 mer oligodeoxynucleotide 5′-GCTAGC**X**AGTCC-3′ (0.03 A_{260} units) in 24 μl ammonium hydrogen citrate buffer (pH 9.4) containing 20 mM $MgSO_4$ was incubated at 37°C with 2 milliunits of snake venom phosphodiesterase I. Aliquots (4 μl) were taken

before enzyme addition and at 1, 8, 18, 28, and 38 min time points after enzyme addition. The aliquots were combined and kept frozen until the analysis. The combined aliquots were desalted using a Millipore C_{18} Ziptips and eluted directly onto a MALDI plate in 3-hydroxypicolinic acid containing ammonium hydrogen citrate (7 mg/ml). MALDI-TOF analysis gave a sequencing ladder from the 3′ → 5′ direction. An identical analysis was performed using 12 milliunits of bovine spleen phosphodiesterase II in 24 μl of 20 mM ammonium acetate buffer (pH 6.6) that provided a complementary sequencing ladder from the 5′ → 3′ direction.

This completes the analysis performed by Dr. Ivan Kozekov.

Construction of circular ss pMS2 DNAs containing the BD N3-dU adducts. The methodologies for introducing site-specific DNA lesions into the ss pMS2 vector (Moriya, 1993) were performed as described by Kanuri et al., (Kanuri et al., 2002a) and Fernandes et al., (Fernandes et al., 2003). In brief, ss pMS2 DNA (59 pmol) was annealed to a 58-mer scaffold (295 pmol), and the annealed product was digested with *Eco*RV to release the hairpin loop structure of ss pMS2 DNA. This procedure results in the creation of a precise gap in the DNA. The scaffolding DNA was designed such that 22 nucleotides on the 5′ end and 24 nucleotides on the 3′ end were complementary to the two termini of the digested ss pMS2. The 58-mer also had a central sequence that was complementary to the 12-mer oligodeoxynucleotide sequence 5′-GCTAGC**X**AGTCC-3′ where **X** is control dC or BD N3-dU. To ensure that scaffolding DNA was not used as a substrate for replication in COS-7 cells, all thymines were substituted by uracil in the 58-mer scaffold. The BD N3-dU 12-mer and the control 12-mer were phosphorylated at the 5′ end using T4 DNA kinase (5 units/pmol) and then ligated (325 units of T4 DNA

ligase/pmol) into the partially duplex pMS2 vector. The ligated DNAs were then purified using Centricon 100 concentrators (to remove unligated 12-mers) and recovered by ethanol precipitation. The products obtained were treated with uracil DNA glycosylase for 1 h to severely damage the scaffold DNA and prevent it from being used as a primer for (-)- strand replication.

Site-specific mutagenesis in COS-7 cells. The overall strategy for replication and mutagenic analyses was as previously described (Fernandes et al., 2003; Kanuri et al., 2002a). COS-7 cells were grown to approximately 70% confluency and transfected with 1 μg of control or BD N3-dU modified DNAs using lipofectin. The transfection medium was replaced after 24 h with DMEM medium containing 10% fetal bovine serum. Following replication for 48 h, the COS-7 cells were harvested and their progeny plasmid recovered as a Hirt supernatant (Hirt, 1967). In order to ensure removal of any input ss DNA and progeny associated with the original ss pMS2 vector, the DNAs so obtained were treated with 1 unit of S1 nuclease in a 60 μl reaction mixture, and with 20 units of *Eco*RV in a 70 μl reaction mixture.

The Hirt supernatant DNAs were used to transform *E. coli* DH5α cells via electroporation and the resultant transformants were selected on LB plates containing 100 μg/ml ampicillin. To detect the presence of mutants, the transformant colonies were individually picked, and grown overnight at 37°C in 96 well plates containing ampicillin supplemented LB medium. These cultures were then transferred, via a 48 pin replicator, onto Whatman 541 filter papers on ampicillin-containing LB plates in 4 replicates (one for each probe), for both the adducts and the control. The *E. coli* on filter papers were then incubated overnight at 37°C to form distinct colonies that were then lysed and

differentially hybridized using the four probes, 5′-GATGCTAGC**N**AGTCCATC-3′, where **N** refers to G, C, A or T.

***In vitro* replication using BD N3-dU containing 32-mer template.** *In vitro* polymerase reactions were carried out using 14-mer oligodeoxynucleotide primers annealed to the template, 5′-ACCATGCCTGCAAGAA**X**TAAGCAATGATCGCC-3′, where **X** represents the site of the BD N3-dU adducts or control dC. Primer oligodeoxynucleotides were first phosphorylated with T4 polynucleotide kinase using [γ-^{32}P] ATP and purified using P-6 Bio-Spin columns supplied with Tris-HCl buffer (pH 7.4). The ^{32}P-labeled primers were then mixed with either adducted or non-adducted template oligodeoxynucleotides at a molar ratio of 1:2 in 25 mM Tris HCl buffer (pH 7.6), 50 mM NaCl, heated at 90°C for 2 min, and cooled to RT overnight. To confirm the completion of primer annealing, aliquots were assayed on a 7.5% native PAGE. The Kf reaction mixture contained 5 nM primer/template DNA in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol and dNTPs at a final concentration of 100 μM and were incubated for 15 min at RT with increasing concentrations (0.0005 to 0.5 units) of the polymerase (where one unit is defined as the amount of enzyme required to convert 10 nmol of dNTPs to an acid-insoluble material in 30 min at 37°C). The Kf exo$^-$ reaction mixture contained 5 nM primer/template DNA in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol and dNTPs at a final concentration of 100 μM and were incubated for 15 min at RT with increasing concentrations (0.0005 to 0.05 units) of the polymerase. The pol δ reaction mixture contained 5 nM primer/template DNA in 40 mM HEPES-KOH (pH 6.8), 10% glycerol, 200 μg/ml bovine serum albumin (BSA), 6 mM $MgCl_2$, 1 mM dithiothreitol, 70 ng calf thymus PCNA and dNTPs at a final

concentration of 100 μM and was incubated for 15 min at RT with increasing concentrations of pol δ from 0.008 to 0.8 units of polymerase. The yeast pol δ reaction mixture contained 5 nM 14-mer primer/template DNA in buffer containing 40 mM Tris HCl (pH 7.8), 0.2 mg/ml BSA, 8 mM Mg Acetate, 0.05 mM ATP and dNTPs at a final concentration of 100 μM and were incubated for increasing time intervals (0, 1, 2, 5, 10, 15, 20 min) at RT with 0.8 nM of the polymerase. The pol ε reaction mixture contained 5 nM primer/template DNA in 50 mM HEPES-KOH (pH 7.5), 100 mM potassium glutamate, 20% glycerol, 200 μg/ml BSA, 15 mM $MgCl_2$, 10 mM dithiothreitol, 0.03% Triton X-100, 100 μM each of the dNTPs, and 0.1 unit of the polymerase. These reactions were carried out for increasing time intervals (0, 1, 5, 10, 15, 20 and 30 min) at RT. Additional reactions were carried out using the same reaction mixture with increasing concentration of the enzyme (0.2, 1, 5 units) for 30 min at RT. For reactions with pol β, the following three substrates were used: 1) primer/template 2) primer/template annealed to a non-phosphorylated complementary oligodeoxynucleotide 3) primer/template annealed to a 5′ phosphorylated complementary oligodeoxynucleotide. The pol β reactions contained 25 mM potassium glutamate, 10% glycerol, 100 μg/ml BSA, 5 mM $MgCl_2$, 5 mM DDT, 5 nM DNA substrate, and 25 nM polymerase. These reactions were incubated at RT for a period of time as indicated in the figure legend. All reactions were terminated by the addition of stop solution consisting of 95% (v/v) formamide, 20 mM EDTA, 0.02% (w/v) xylene cyanol, and 0.02% (w/v) bromphenol blue. Reaction products were resolved on a 15% denaturing PAGE and visualized by autoradiography. Quantitative analyses were performed using a PhosphorImager screen and Image-Quant software.

RESULTS

Synthesis and characterization of the BD N3-dU containing oligodeoxynucleotides. Standard protecting group chemistries were utilized in the formation of the 2′-deoxyuridine precursor **2** for alkylation (Scheme 2.1). For the synthesis of the buten-ol alkylating reagent **4** (Scheme 2.2), the C1 alcohol was selectively protected in high yield as the p-toluenesulfonate ester **3** using the alpha-chelating method of Martinelli, et al. (Martinelli et al., 2002). The toluenesulfonate group served as both protections for subsequent silylation of the C2 alcohol to give **4** and as the leaving group for the alkylation reaction at N3 of 2′-deoxyuridine. However, the alkylation reaction of **2** under aprotic conditions was very slow and did not go to completion (47%) (Scheme 2.3). Even after 8 days, unreacted starting materials were still recoverable. The p-toluoyl group of the C-3′ alcohol of alkylated product **5** was readily removed by a protic solution of KOH, and the resulting product **6** was then phosphitylated using the bis-N,N,N′N′-tetraisopropyl-2-O-cyanoethyl-phosphine procedure to give the required phosphoramidite **7** in good yield and purity for DNA synthesis. HPLC analysis of the 12-mer dU-BuOSi oligodeoxynucleotide showed two distinct overlapping species; however, they could not be independently isolated upon further purification. Subsequently, the silyl protecting group was removed by fluoride treatment and Nap-10 purification to give the BD N3-dU 12-mer oligodeoxynucleotide that was used as the *R, S* mixture in all further experiments. HPLC analysis of the 32-mer dU-BuOSi oligodeoxynucleotide also showed two distinct overlapping species; however, these could not be independently isolated upon further purification. Subsequently, this product was treated and purified as above to remove the silyl protecting group and used as the *R, S* mixture in all further experiments.

The 12-mer adducted oligodeoxynucleotide used for the construction within the pMS2 shuttle vector was analyzed by CGE and judged to be >96 % pure (Fig. 2.1A). Under the CGE conditions used, the diastereomers could be partially resolved. Enzymatic digestion of the oligodeoxynucleotide using a mixture of DNase I, snake venom phosphodiesterase I, and alkaline phosphatase, followed by HPLC analysis showed the appropriate nucleosides in the correct molar ratios (Fig.2.1B). The modified nucleoside was identified by comparison of the retention time with an authentic sample and by co-injection.

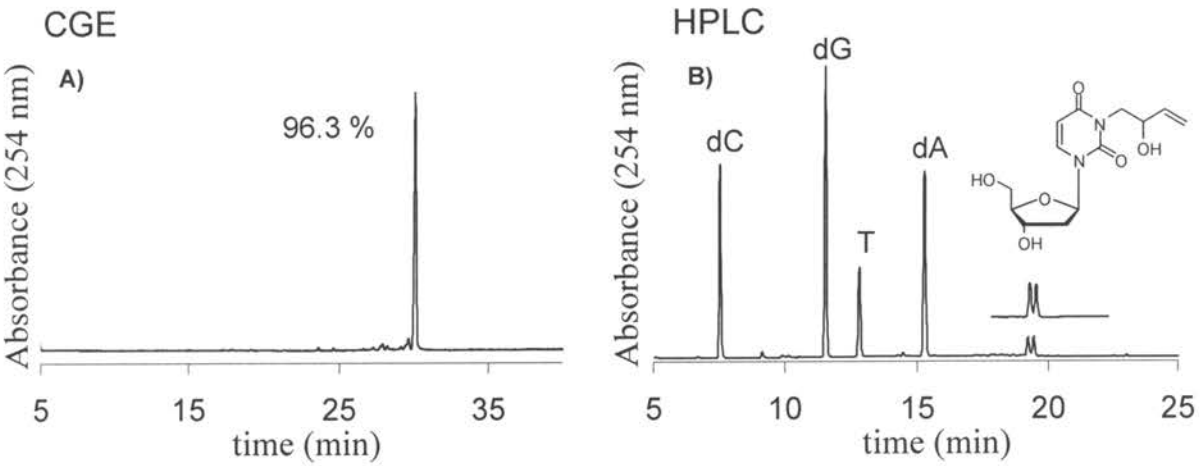


Fig. 2.1. Analysis of 12-mer oligodeoxynucleotide 5′-GCTAGC**X**AGTCC-3′. (A) Capillary gel electrophoresis profile. (B) HPLC profile of the enzymatic hydrolysate. The HPLC trace of an authentic sample of the adducted nucleoside is superimposed.

The oligodeoxynucleotide was then sequenced by partial enzymatic digestion with snake venom phosphodiesterase I. Aliquots were taken at various time points, combined and then analyzed by MALDI-TOF mass spectrometry. A sequencing ladder was obtained as a result of sequential loss of the nucleotides from the 3′ → 5′ direction (Tretyakova et al., 2001) (Fig. 2.2A). For phosphodiesterase I, the digestion was arrested at the adducted nucleotide. A complementary analysis was performed using bovine spleen phosphodiesterase II to determine the sequence from the 5′ → 3′ direction (Fig. 2.2B).

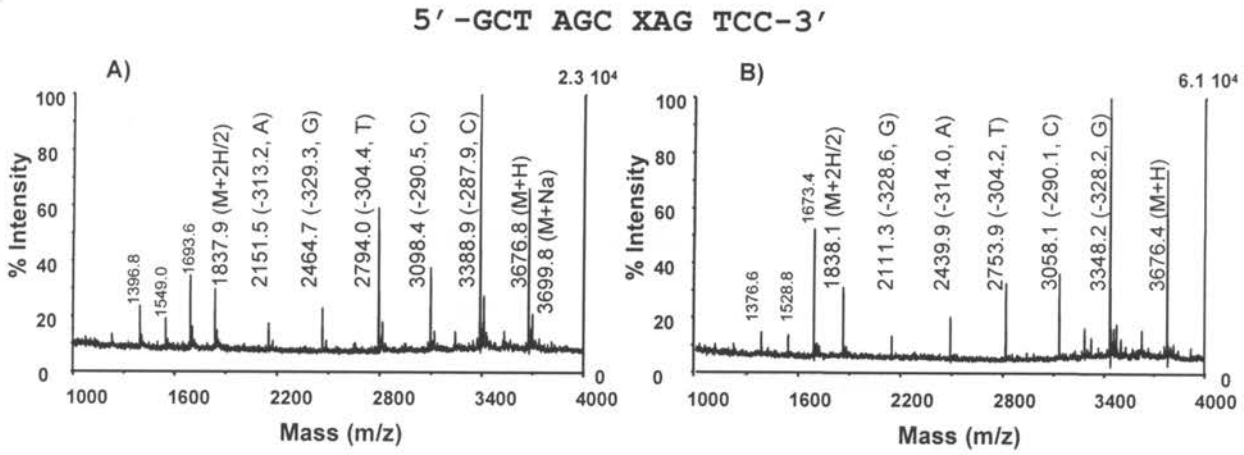


Fig. 2.2. MALDI-TOF analysis of partial exonuclease digestion of 12-mer oligodeoxynucleotide 5′-GCTAGC**X**AGTCC-3′. (A) 3′→ 5′ sequencing using phosphodiesterase I. (B) 5′→ 3′ sequencing using phosphodiesterase II.

In this analysis, the progress of the phosphodiesterase II was halted one base before the adducted nucleotide. Although the nucleases were unable to digest through the adducted nucleotide on the time scale of these analyses, the results are consistent with the sequence noted.

Replication and Mutagenesis of DNA containing the site-specific N3-dU adducts of butadiene. The single stranded pMS2 shuttle vector was annealed to a scaffold and digested with *Eco*RV to create a gap into which the BD N3-dU adducted or control dC oligodeoxynucleotide was ligated, followed by digestion of the scaffold. These modified vectors were replicated in COS-7 cells and the resultant progeny DNAs were transformed into DH5α *E. coli* cells. There was no appreciable difference in the absolute number of transformants between the adducted and control DNAs, implying that these lesions may not pose total blocks to replication within mammalian cells. These transformants were subjected to differential hybridization (Fernandes et al., 2003; Kanuri et al., 2002a) using one of the four probes (5′-GATGCTAGC**N**AGTCCATC-3′ where **N** refers to G, C, A or T). Typical autoradiographic results are shown in Fig. 2.3.

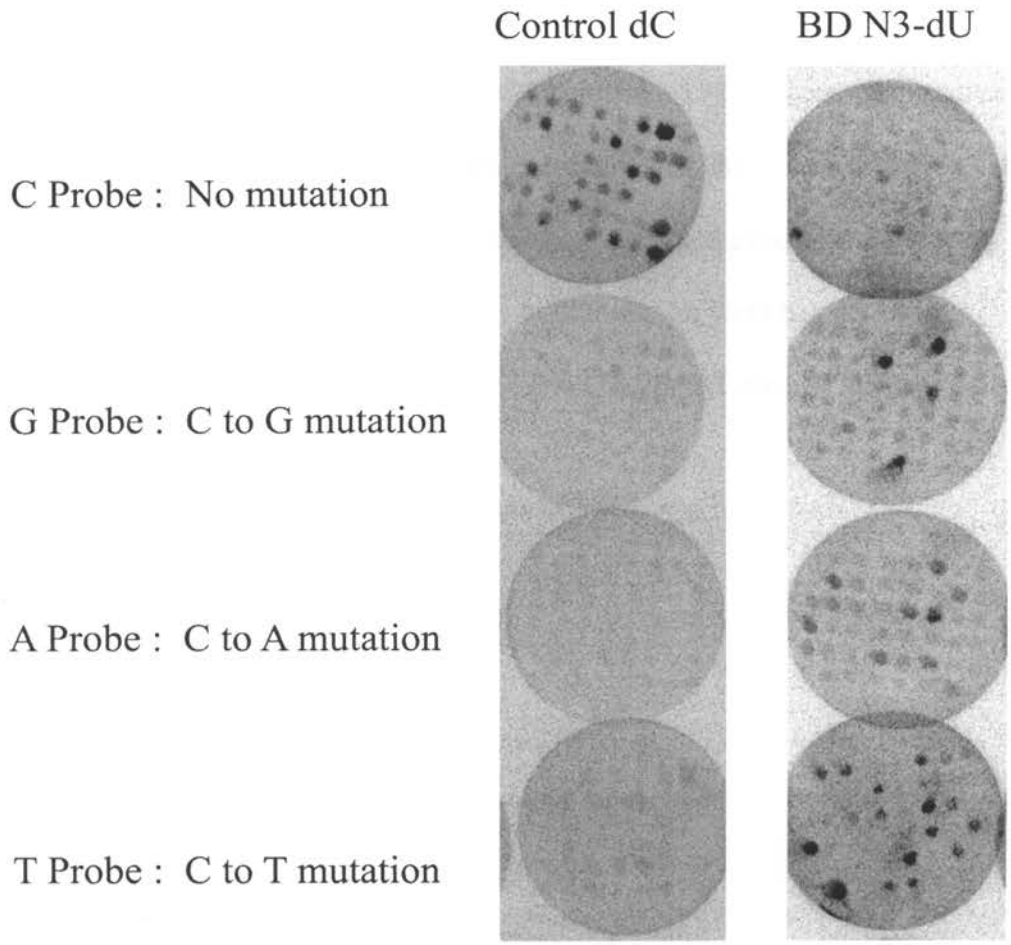


Fig. 2.3. Autoradiographs displaying the result of mutagenic replication past the BD N3-dU adducts in COS-7 mammalian cells. Single-stranded pMS2 vector containing the BD N3-dU adducts or control dC was transfected into COS-7 cells and replicated for 48 h. The progeny plasmids were then recovered from the COS-7 cells and used to transform DH5α E. *coli* cells. Individual transformants were picked and grown in 96-well plates containing LB with ampicillin. The resultant colonies were transferred in four replicates onto Whatman 541 filter papers, one for each of the four probes G, C, A, and T, and were differentially hybridized.

The intensely hybridizing cells were scored as positive colonies that contained the progeny DNAs derived from the replication of adduct-containing or control sequences. All experimental procedures were repeated in triplicate and ~ 190 colonies were analyzed for both adduct-containing and control oligodeoxynucleotides. The overall mutation yield for the BD N3-dU adducts was around 97%, while no mutational events were detected with the control dC (Table 2.1). This remarkably high mutation yield has not been observed with any other butadiene DNA adduct except for the N1 inosine DNA adducts (Kanuri et al., 2002b)

Single-stranded vector pMS2 containing	No. of colonies	Non mutagenic (%)	C to G (%)	C to A (%)	C to T (%)	Total Mutation Yield (%)
Control dC	199	100	0	0	0	0
BD N3-dU adducts	191	3.1	11	32.5	53.4	96.9

Table 2.1. Mutation Yield of the BD N3-dU adducts in COS-7 mammalian cells

The predominant mutations were C to T transitions (53.4%) and C to A transversions (32.5%), followed by a much lower frequency of C to G transversions (11%). To verify the correct identification of mutant sequences derived by the differential hybridization method, random colonies that hybridized to specific probes were sequenced using a primer located upstream from the original adducted site. The sequencing data confirmed that the base substitution mutations detected by autoradiography were properly identified and these data did not reveal any alternate replication bypass events such as deletions or frameshifts. These analyses indicate that these lesions primarily (if not exclusively) induce base substitution mutations at a very high rate.

***In vitro* replication.** To assess the effect of the BD N3-dU lesions on *in vitro* replication, a labeled 14-mer primer was annealed to the 32-mer template containing the BD N3-dU adducts or control dC and several polymerases assayed for their ability to replicate past these lesions. The location of the 3′ OH was such that the polymerases would incorporate one nucleotide prior to incorporation opposite the lesion. Replication past the adducts was first tested with Kf, a model polymerase. On the nondamaged DNA substrate, an efficient extension of the primers was observed (Fig. 2.4, lanes 2-5). In contrast, on the BD N3-dU containing substrate, after one nucleotide was incorporated, indicating efficient loading and polymerization further synthesis was greatly reduced, suggesting that the BD N3-dU adducts posed a near complete block to further replication. No formation of the full-length size products was detected (Fig. 2.4, lanes 7-10). Even in the case when Kf exo$^-$ was used, majority of the DNA synthesis was blocked 1 nucleotide prior to the adducts, with minimal incorporation opposite the adducts as seen in Fig 2.5. Therefore the BD N3-dU adducts pose a major block to Kf.

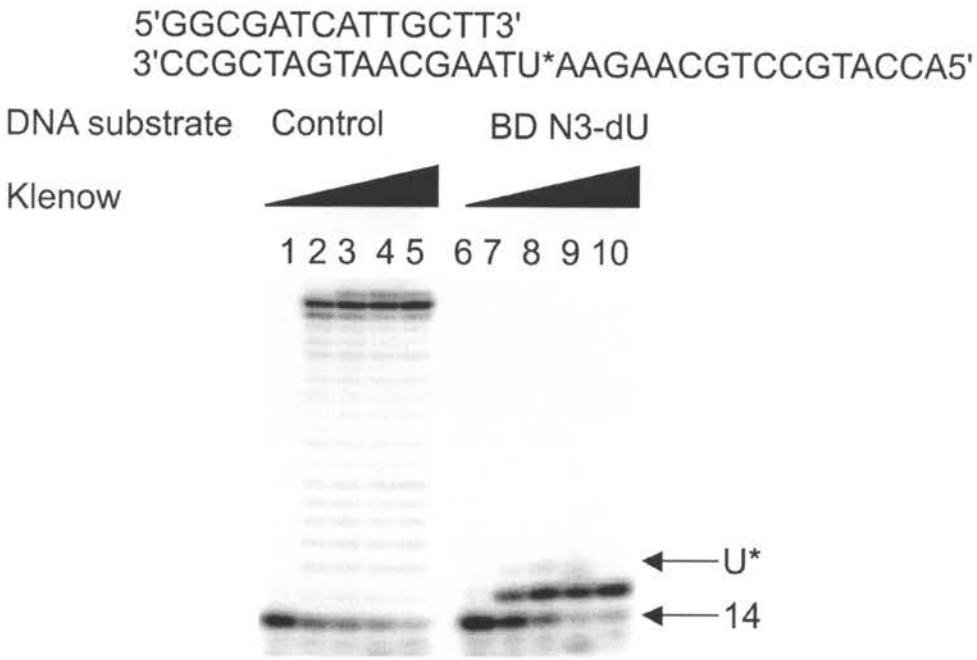


Fig. 2.4. Primer extension reactions catalyzed by Kf on the BD N3-dU adducted template. Control dC or BD N3-dU adducted 32-mer DNA templates were annealed to a -2 primer. The DNA substrates (5 nM) were incubated in the presence of all four dNTPs (100 μM) without or with increasing concentrations (0.0005, 0.005, 0.05 and 0.5 units) of Kf (lanes 2-5 and 7-10) for 15 min at RT. The position of the 14-nucleotide primer is indicated. U* indicates the position of the modified uracil on the template.

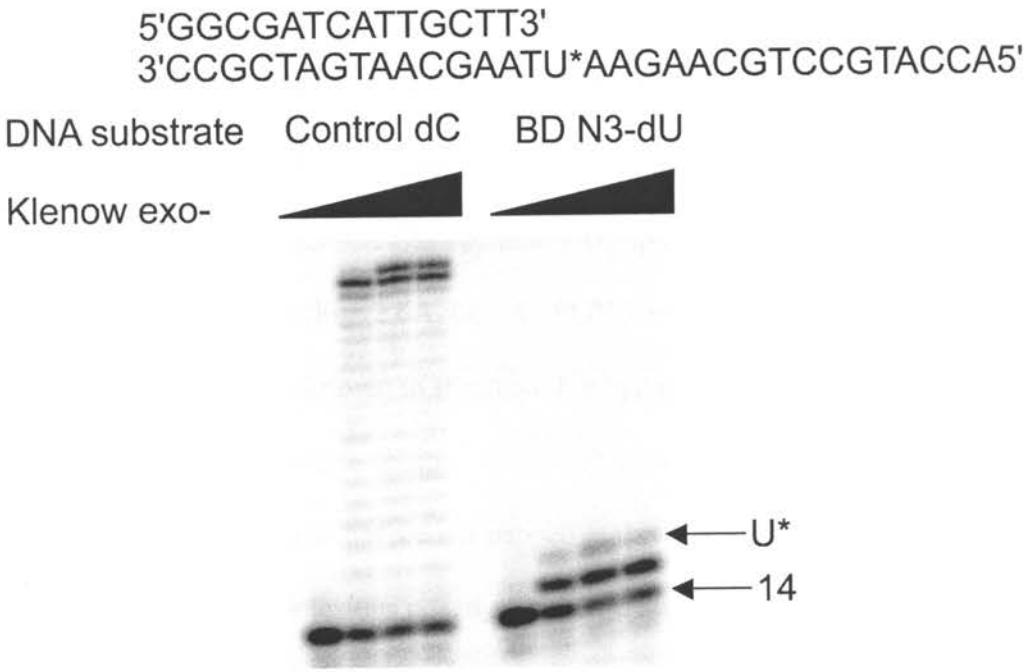
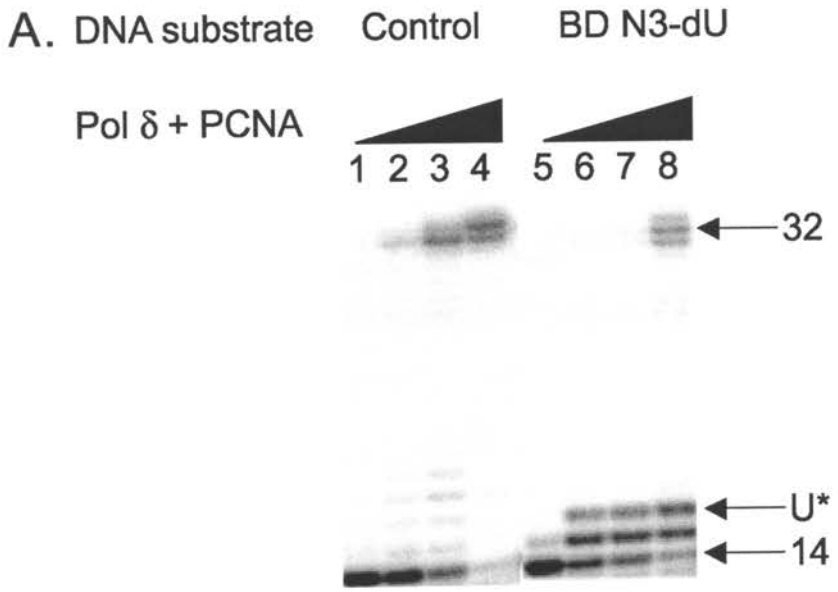


Fig. 2.5. Primer extension reactions catalyzed by Kf exo⁻ on the BD N3-dU adducted template. Control dC or BD N3-dU adducted 32-mer DNA templates were annealed to a -2 primer. The DNA substrates (5 nM) were incubated in the presence of all four dNTPs (100 μM) without or with increasing concentrations (0.0005, 0.005, 0.05 units) of Kf exo⁻ for 15 min at RT. The position of the 14-nucleotide primer is indicated. U* indicates the position of the modified uracil on the template.

Mammalian replicative polymerases δ and ε were also examined for their ability to bypass these lesions. In the presence of pol δ and PCNA, the primer was extended by one nucleotide but further polymerization opposite the BD N3-dU adducts was greatly reduced (Fig. 2.6A, lanes 6-8). However, partial bypass of the lesions was observed (Fig. 2.6A, lane 8) under conditions that allowed nearly complete replication of the nondamaged DNA template (Fig. 2.6A, lane 4). PCNA was added to this reaction since it is a pol δ processivity factor known to stimulate the bypass of certain DNA lesions (γ-hydroxypropano deoxyguanosine (Kanuri et al., 2002a) and thymine dimers (O'Day et al., 1992) even on DNA templates that are not capped at their termini to restrict the release of the PCNA. Interestingly, the lesions posed an extreme block for replication by yeast pol δ (Fig. 2.7). In kinetic experiments using pol ε, primer extension was completely blocked one nucleotide prior to and at the site of the lesions under conditions that supported polymerization on unmodified templates (Fig. 2.6B). The same results were observed with increasing concentrations of pol ε that enabled complete polymerization on the unmodified templates (Fig. 2.8). Therefore replicative polymerases pol δ and pol ε are severely blocked by the presence of the BD N3-dU adducts.

5'GGCGATCATTGCTT3'
3'CCGCTAGTAACGAATU*AAGAACGTCCGTACCA5'



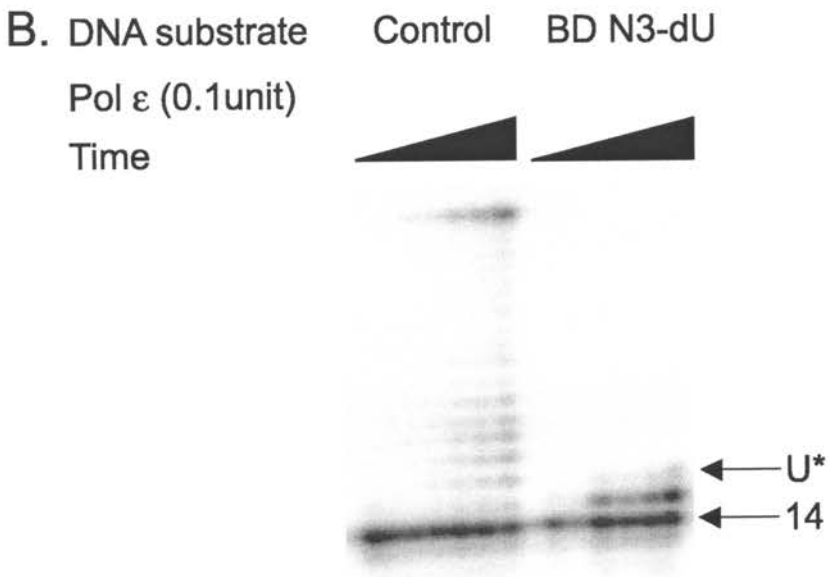


Fig. 2.6. Primer extension reactions catalyzed by calf thymus pol δ and human pol ε on the BD N3-dU adducted template. Control dC or BD N3-dU adducted 32-mer DNA templates were annealed to a -2 primer. (A) The DNA substrates (5 nM) were incubated in the presence of all four dNTPs (100 μM) without or with increasing concentrations (0.008, 0.08 and 0.8 units) of pol δ, in the presence of 70 ng of calf thymus PCNA for 30 min at RT. (B) Time course experiments (at time intervals of 0, 1, 5, 10, 15, 20 and 30 min) were carried out with the DNA substrates (5 nM) at RT in the presence of all four dNTPs (100 μM) with 0.1 units of pol ε. The positions of the 14-nucleotide primers and the 32-nucleotide full-length products are indicated. U* indicates the position of the modified uracil on the template.

5'GGCGATCATTGCTT3'
3'CCGCTAGTAACGAATU*AAGAACGTCCGTACCA5'

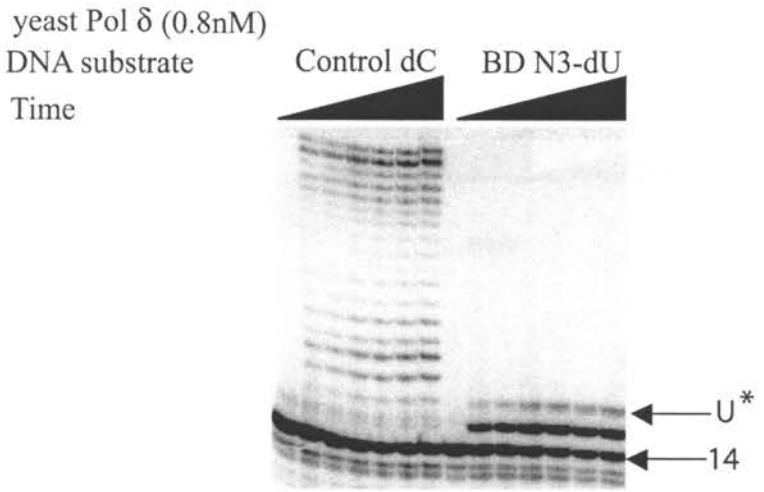


Fig. 2.7. Primer extension reactions catalyzed by yeast pol δ on the BD N3-dU adducted template. Control dC or BD N3-dU adducted 32-mer DNA templates were annealed to a -2 primer. Time course experiments (at time intervals of 0, 1, 2, 5, 10, 15, and 20 min) were carried out with the DNA substrates (5 nM) at RT in the presence of all four dNTPs (100 μM) with 0.8 nM of the polymerase. The positions of the 14-nucleotide primers are indicated. U* indicates the position of the modified uracil on the template.

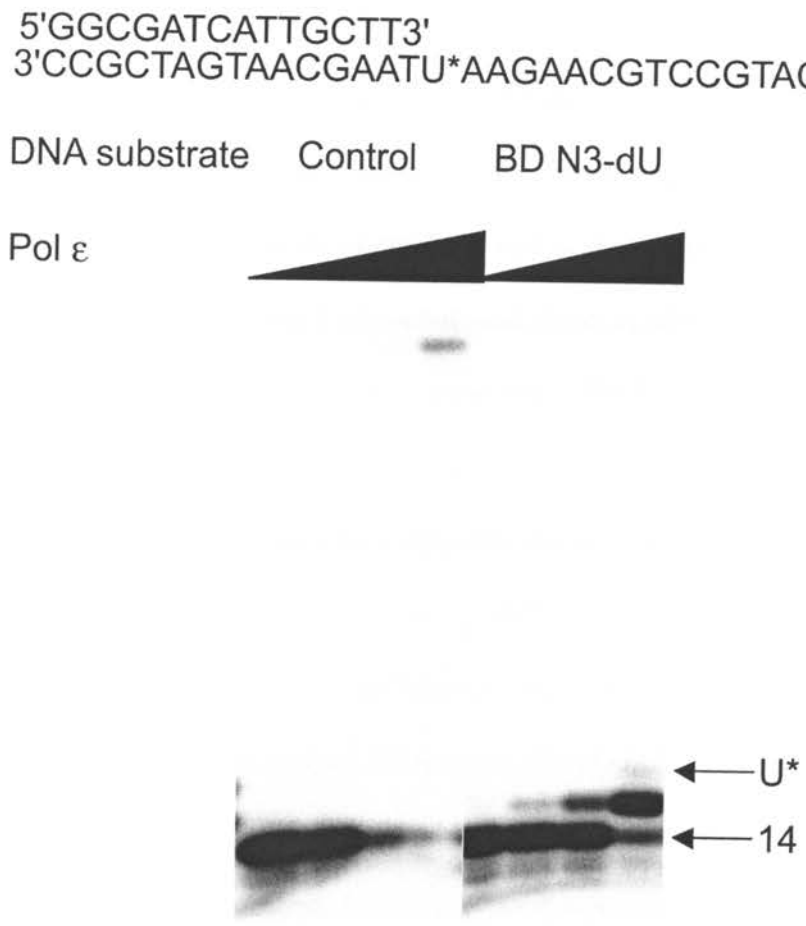


Fig. 2.8. Primer extension reactions catalyzed by human pol ε on the BD N3-dU adducted template. Control dC or BD N3-dU adducted 32-mer DNA templates were annealed to a -2 primer. The DNA substrates (5 nM) were incubated in the presence of all four dNTPs (100 μM) without or with increasing concentrations (0.2, 1 and 5 units) of pol ε for 30 min at RT. The positions of the 14-nucleotide primers are indicated. U* indicates the position of the modified uracil on the template.

DNA pol β, in addition to its function in base excision, is known to bypass cyclobutane pyrimidine dimers, cisplatin- and oxaliplatin- GG adducts and the 8-(hydroxymethyl)-3,N(4)-etheno-C adduct (Hoffmann et al., 1995; Servant et al., 2002; Singer et al., 2002; Vaisman and Chaney, 2000). It shows optimum activity when provided with a gapped substrate where the 5′ end of the gap is phosphorylated. The 8 kD domain of pol β can bind to the 5′ phosphate and direct synthesis through the gap with its 31 kD domain (Chagovetz et al., 1997; Prasad et al., 1994; Singhal and Wilson, 1993). Accordingly, to assess the effect on pol β, three substrates were designed for the control and BD N3-dU adducts (A = a primed template, B = a primed template with a non-phosphorylated downstream oligodeoxynucleotide and C = a primed template with a 5′ phosphorylated downstream oligodeoxynucleotide). Under conditions that allowed primer extension on the undamaged DNA substrate A (Fig. 2.9A, lane 2) DNA synthesis by pol β was blocked on the BD N3-dU adducted substrate A (Fig. 2.9B, lane 8). Similarly, on the gapped substrate B in which the downstream DNA fragment was not phosphorylated, pol β synthesis was arrested one nucleotide preceding the lesion (Fig. 2.9A, lane 10). However, pol β exhibited limited gap-filling and lesion bypass activity on substrate C, in which a 5′ phosphorylated oligodeoxynucleotide was present downstream to the primer (Fig. 2.9A, lane 12). Using this substrate, time course experiments were then carried out. Partial gap-filling and lesion bypass by pol β was observed in 5 min. However, for the same time period, strand displacement had already occurred on the undamaged DNA substrate (Fig. 2.9B). Therefore pol β is most likely not responsible for the bypass of the BD N3-dU adducts under physiological conditions.

Substrate A = 5'GGCGATCATTGCTT3'
3'CCGCTAGTAACGAATU*AAGAACGTCCGTA

Substrate B = 5'GGCGATCATTGCTT CTTGCAGGCAT3'
3'CCGCTAGTAACGAATU*AAGAACGTCCGTA5'

Substrate C = 5'GGCGATCATTGCTT $^{5'P}$CTTGCAGGCAT3'
3'CCGCTAGTAACGAATU*AAGAACGTCCGTA5'

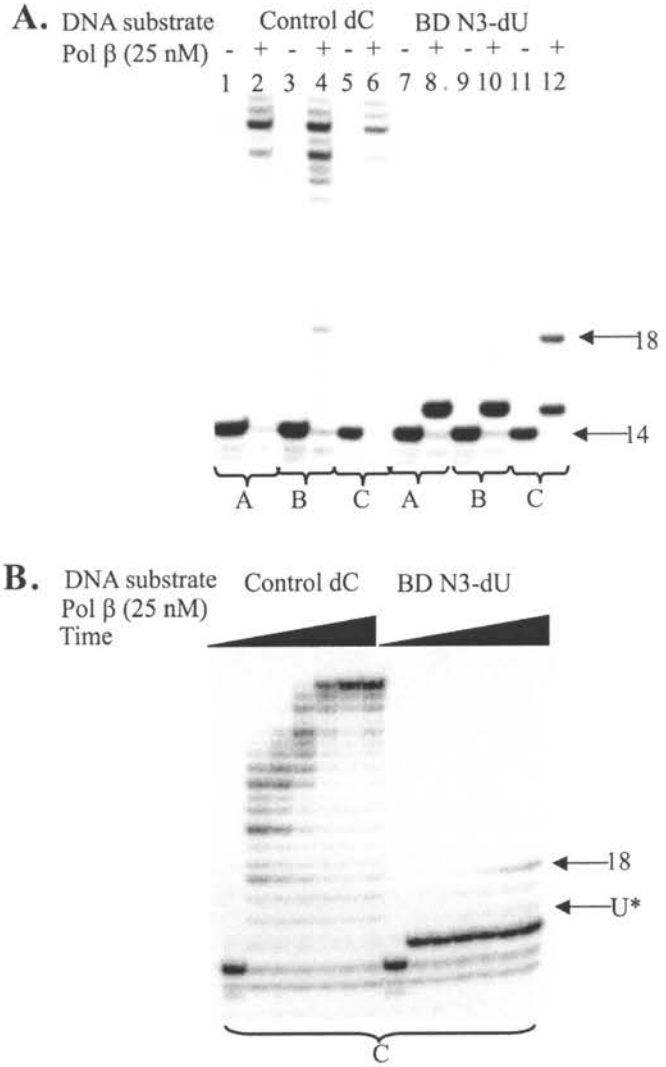


Fig. 2.9. Primer extension reactions catalyzed by human pol β on the BD N3-dU adducted template. Substrate A, B or C were used in the following reactions. (A) Theses DNA substrates (5nM) were incubated for 30 min at RT in the presence of all four dNTPs (100μM) without (-) or with (+) 25nM of DNA polymerase β (lanes 1-12). (B) Kinetic experiments (at time intervals 0, 0.5, 1, 2, 5, 10, 15 min) were carried out using substrate

C in the presence of all four dNTPs (20μM) with 25nM of DNA pol β. The positions of the 18-nucleotide gap filled primers are indicated. U* indicates the position of the modified uracil on the template.

DISCUSSION

The BD N3-dU adducts are highly mutagenic but are severe blocks to various polymerases like Kf, pol δ, pol ε and pol β. Research in BD-mediated carcinogenesis has included the identification of those DNA adducts that may induce mutagenesis. Site-specific methodologies have made it possible to evaluate the effect of individual DNA adducts on replication as compared to general BD exposure studies. Of the various BD-derived DNA monoadducts analyzed, the two most highly mutagenic adducts were the N1-inosine adducts (Kanuri et al., 2002b) and the BD N3-dU adducts, as shown in this study. The N1-inosine adducts, like the N3-dU adducts are deamination products following epoxidation by EB (Selzer and Elfarra, 1996a).

Nucleotide incorporation studies using exonuclease-deficient Kf with N3-hydroxyethyl uracil have revealed this adduct to be mutagenic, implicating it in the induction of mainly C to T transitions and C to A and C to G transversions to a minor extent (Zhang et al., 1995a). This is similar to the trend of mutations measured with the BD N3-dU adducts (Table 2.1). These data suggest that adduction at the N3 position of uracil renders it mutagenic irrespective of the exact identity of the parent epoxide. Structural data on the N3 BD-dU adducts are not yet available, but would give a better understanding of the relationship of the positioning of this adduct within DNA to explain the incorporation of the incorrect nucleotides opposite these lesions during replication in cells. This has been demonstrated in the case of other adducts of BD like the N1 deoxyinosine adducts (Merritt et al., 2005; Scholdberg et al., 2005) in which the *syn* conformation of the adducts at the glycosyl bond could explain its induction of A to G mutations.

Assays to detect the formation of BD N3-dU adducts following *in vivo* exposure to butadiene gas or activated metabolites have not yet been carried out and thus, it is an open question whether these lesions form at an appreciable rate in humans. It is reasonable to speculate that they may be present at low levels as was seen in the case of 3-hydroxypropyl uracil, the only uracil DNA lesion to be detected *in vivo*. This adduct was found at 0.02 lesions/10^6 nucleotides and at 0.02% of the corresponding 7-hydroxypropyl guanine (Plna et al., 1999). However, the data presented herein on the extremely high mutagenic frequency associated with the replication of these lesions suggest that such an investigation is well warranted.

The BD N3-dU adducts are formed from the N3 (2-hydroxy-3-buten-1-yl) deoxycytidine adducts that are unstable under physiological conditions. The deoxycytidine adducts have a high deamination rate with short half-lives of 2.3 and 2.5 h, whereas their deaminated products are stable for up to 168 h (Selzer and Elfarra, 1997b). Similarly 3-(2-hydroxyethyl)-2'-deoxycytidine derived from bis(2-chloroethyl)nitrosourea is unstable under physiological conditions and undergoes deamination to 3-(2-hydroxyethyl)-2'-deoxyuridine with a half-life of approximately 5 h (Zhang et al., 1995b). Thus these uracil adducts are readily formed and may remain persistent in DNA. Other epoxides which yield a uracil product on reaction with cytidine or calf thymus DNA include propylene oxide (Solomon et al., 1988), ethylene oxide (Li et al., 1992), 2-cyanoethyleneoxide (Solomon et al., 1993), acrylonitrile, (Solomon and Segal, 1989)epichlorohydrin, (Singh et al., 1996) and glycidol (Segal et al., 1990). It is suggested that once the epoxide attacks the N3 of deoxycytidine, the hydroxyalkyl side chain intramolecularly catalyzes the hydrolysis of the adjacent imine C=NH, bond. As a

result an unstable carbinolamine intermediate is formed that loses ammonia to form the uracil adduct (Solomon and Segal, 1989).

To date there is no information on the repair of the BD N3-dU adducts. *In vitro* assays utilizing BER glycosylases like UDG, SMUG1 and Endo III did not result in excision of these adducts (Figs. 2.10-2.12). Nucleotide excision repair may be involved in their repair as evidenced by sensitivity of XPC null mice to EB (Wickliffe et al., 2006), the same metabolite from which the BD N3-dU adducts are derived.

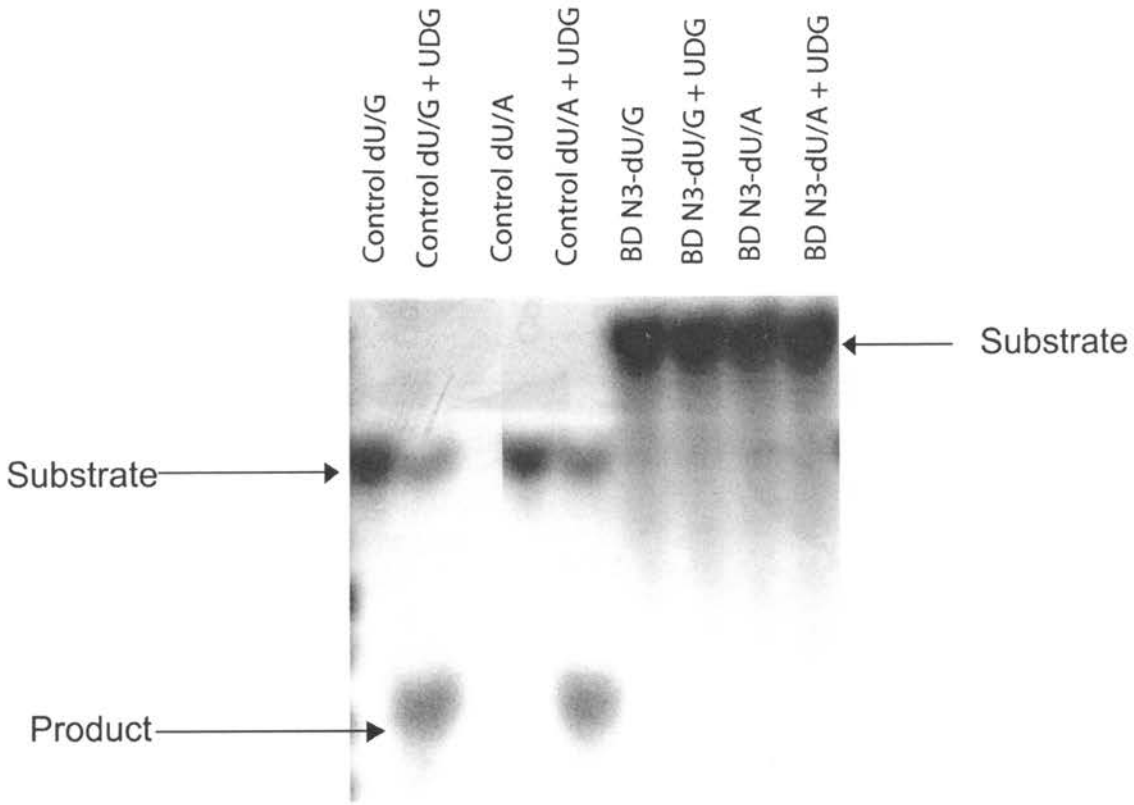


Fig. 2.10. Activity of UDG on the BD N3-dU adducted template. The 5′ labeled oligodeoxnucleotide substrates (Control dU/G, Control dU/A, 32BD N3-dU/G, 32BD N3-dU/A) (5nM) were incubated with 0.2 units of UDG at 37°C for 30 min in a buffer containing 20 mM Tris-HCl, 1 mM dithiothreitol, 1 mM EDTA (pH 8). The reaction was terminated by addition of formamide containing 0.5 N NaOH.

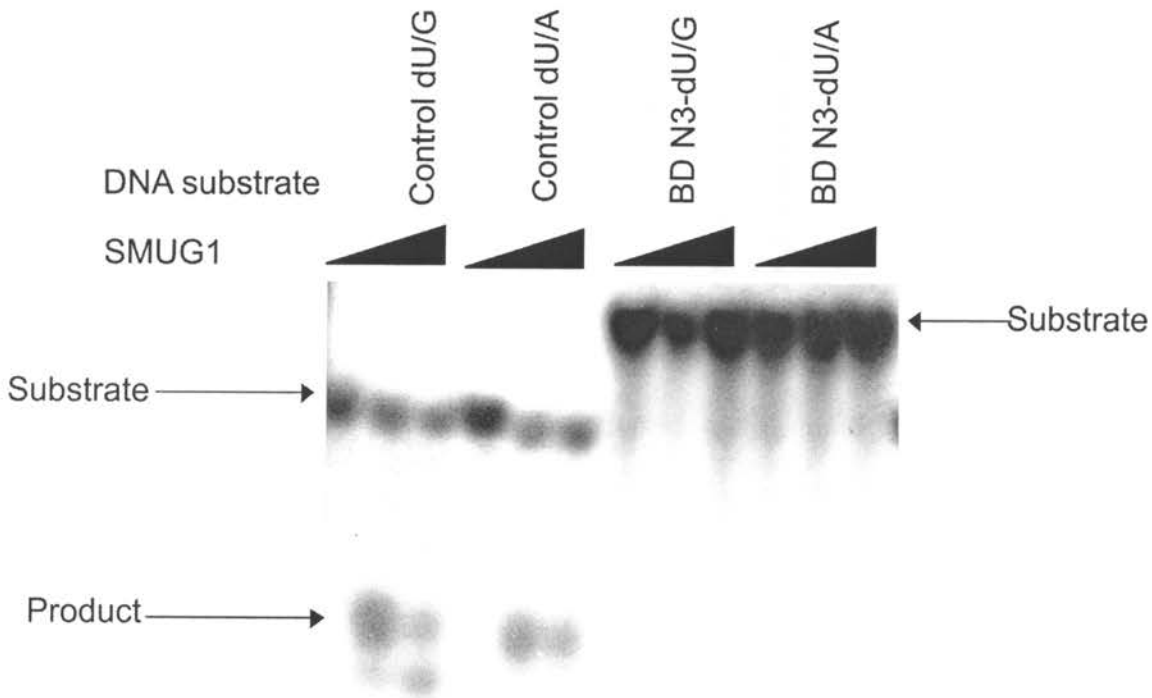


Fig. 2.11. Activity of SMUG1 on the BD N3-dU adducted template. The 5′ labeled oligodeoxynucleotide substrates (Control dU/G, Control dU/A, 32BD N3-dU/G, 32BD N3-dU/A) (5nM) were incubated with increasing concentrations of the SMUG1 (1.47 μg, 4.4 μg) at 37°C for 30 min in a buffer containing 20 mM Tris-HCl, 1 mM dithiothreitol, 1 mM EDTA (pH 8). The reaction was terminated by addition of formamide containing 0.5 N NaOH.

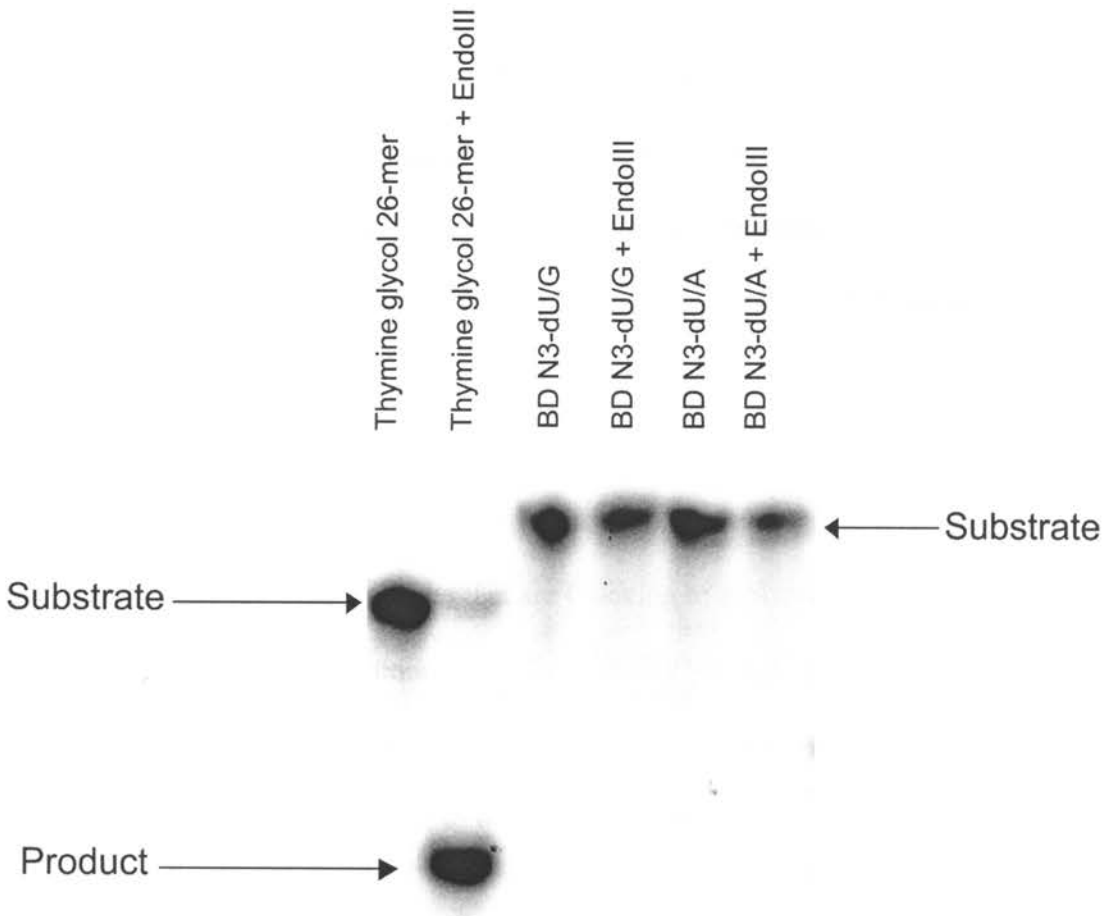


Fig. 2.12. Activity of Endo III on the BD N3-dU adducted template. The 5′ labeled oligodeoxynucleotide substrates (thymine glycol 26-mer, 32BD N3-dU/G, 32BD N3-dU/A) (5nM) were incubated with 2 units of the enzyme at 37°C for 1 hr in a buffer containing 20 mM Tris-HCl, 1 mM dithiothreitol, 1 mM EDTA (pH 8). The reaction was terminated by addition of formamide

My studies indicate that mammalian pol ε and bacterial Kf exo$^+$ and exo$^-$ are severely blocked upon encountering the BD N3-dU adducts. They also pose as blocks to pol δ and pol β, although some bypass activity was observed. *In vivo*, these adducts may be recognized and bypassed more efficiently by specialized translesion polymerases like polymerases η, ι, ζ, and κ that are implicated in the bypass of certain DNA adducts (Johnson et al., 2000b; Masutani et al., 2000; Washington et al., 2004c; Zhang et al., 2000b).

In summary, we have synthesized and established the mutagenic effect of the BD N3-dU adducts. They are stable adducts that are blocking to replicative and repair polymerases and mutagenic in mammalian cells probably through replication by translesional polymerases. These data thus suggest the importance of the BD N3-dU adducts as crucial lesions contributing to BD-carcinogenesis.

ACKNOWLEDGEMENTS

I acknowledge Dr. Masaaki Moriya, Dr. Kathleen M. Downey, Dr. Hitomi Asahara and Dr. Stuart Linn, and Dr. Peter M. Burgers, for the generous gifts of ss pMS2, pol δ, pol ε, and yeast DNA pol δ, respectively. We thank Dr. Irina G. Minko for helpful discussions and Dr. Carmelo Rizzo for his efforts in facilitating the characterization of the modified oligodeoxynucleotides.

CHAPTER 3

MUTAGENIC BYPASS OF THE BUTADIENE-DERIVED N3 2′-DEOXYURIDINE ADDUCTS BY POLYMERASES ETA AND ZETA

ABSTRACT

The N3 2′-deoxyuridine adducts represent one of the major stable pyrimidine adducts that originate from cytidine adducts formed from the monoepoxide metabolite of butadiene. In the previous chapter, it was shown that replication of DNAs containing site-specific N3 2′-deoxyuridine adducts through mammalian cells resulted in ~97% mutagenicity, with the predominant mutation being C to T transitions. These adducts also posed major blocks to replicative polymerases delta and epsilon *in vitro*. Since the major replicative polymerases were blocked by these lesions, translesional polymerases ι, κ, η and ζ were assessed for their ability to bypass these adducts. While polymerases iota, kappa and zeta were significantly blocked one nucleotide prior to the lesion, pol eta incorporated nucleotides opposite the adducts, with a preference for insertion of G or A. Pol eta was also able to extend primers with mispaired termini opposite the lesions. Extension from the A and T mismatched primer termini was the most efficient. In pol-eta catalyzed replication both the incorporation and extension steps required more than one encounter with the polymerase. Pol zeta was also able to extend primers containing all mismatched nucleotides opposite the lesions, with the most efficient extension occurring off of the A mismatched terminus primer. *In vitro* data demonstrate a potential synergistic effect between pol eta and pol zeta in the bypass of these adducts. Thus, pol eta and pol zeta may be involved in the induction of C to T mutations in mammalian cells.

INTRODUCTION

The initiation and progression of human neoplasias is a multifactorial process that encompasses the interplay of both genetic susceptibilities and environmental toxicant exposures. Key to establishing linkages between genotoxic exposures and increased cancer rates are large-scale epidemiological analyses. Such a positive correlation has been made for BD that is both a high volume industrial chemical used in the rubber industry and a common environmental contaminant (Brunnemann et al., 1990; Pelz et al., 1990). The uptake of butadiene in humans occurs almost exclusively by inhalation and absorption through the respiratory tract, thus making individuals readily susceptible to its effects. In humans, the lympho/haematopoetic system is considered to be the target site for BD, since epidemiological studies indicate an increased risk of leukemia and lymphomas to occupational workers exposed to this chemical (Delzell et al., 1996; Graff et al., 2005; Macaluso et al., 1996; Santos-Burgoa et al., 1992). Although BD has been classified as a probable human carcinogen (IARC, 1999), the exact mechanisms underlying BD-mediated mutagenesis and carcinogenesis are not well understood. Additional studies have established BD to be a multi-site rodent carcinogen, with mice being more sensitive than rats (Melnick et al., 1990; Owen et al., 1987).

Several BD-derived monoadducts have been characterized following treatment of DNA *in vitro,* as well as in DNA isolated from exposed organisms. Some of these include the N1, N^2, and N7 adducts of deoxyguanosine (Boogaard et al., 2001; Powley et al., 2005; Selzer and Elfarra, 1996b; Zhang and Elfarra, 2004), the N1, N3, N^6 adducts of adenine (Selzer and Elfarra, 1996a; Selzer and Elfarra, 1999; Tretyakova et al., 1998; Zhao et al., 2000) and the N3 adducts of cytidine and thymidine (Selzer and Elfarra,

1997a; Selzer and Elfarra, 1997b). Of particular interest are the BD N3-dU adducts. These adducts are formed when EB reacts with cytidine or calf thymus DNA to form the N3 deoxycytidine adducts which are unstable with a half life of less than 3 h (Selzer and Elfarra, 1997b). They undergo hydrolytic deamination to give rise to the BD N3-dU adducts (Fig. 3.1) that are highly stable (Selzer and Elfarra, 1997b) and can remain persistent in DNA long enough to cause mutations.

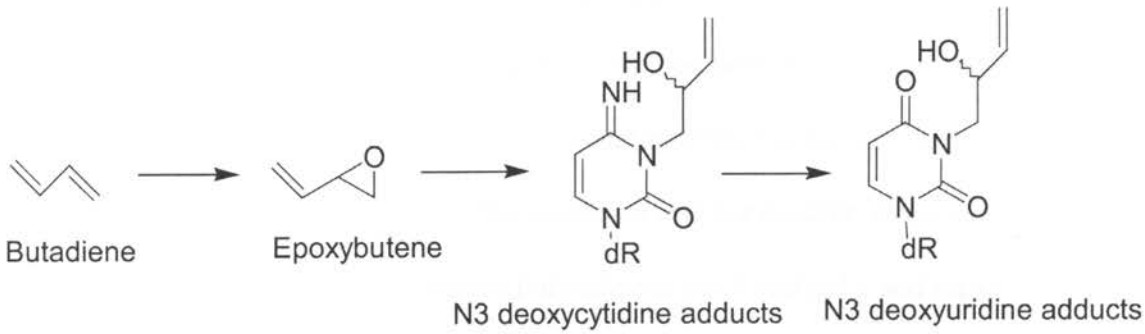


Fig 3.1. Formation of the BD N3-dU adducts. BD is metabolically activated to EB that can react with cytidine to form the N3 cytidine adducts. These adducts are unstable and undergo hydrolytic deamination to give rise to the stable BD N3-dU adducts.

In the previous chapter, we described the synthesis of the BD N3-dU adducts, their site-specific inocorporation into oligodeoxynucleotides and their mutagenic potential in mammalian cells. Replication of vectors containing these adducts was found to be ~97 % mutagenic, with the majority of the mutations being C to T and C to A mutations. Although these lesions could be bypassed in COS-7 cells, the identity of candidate polymerase(s) that carried out this synthesis was not evident, since these adducts were highly blocking to replicative polymerases pol δ and pol ε and repair polymerase pol β. Therefore in this study, we have examined the ability of several translesional DNA polymerases (human pol ι, pol κ, pol η and yeast pol ζ) to bypass these adducts. A model for the bypass of the BD N3-dU adducts involving pol η alone or pol η and pol ζ is proposed. Additionally, this is the first report of an *in vitro* synergistic action between pol η and pol ζ to mediate bypass of these DNA adducts.

MATERIALS AND METHODS

Reagents. Human DNA polymerases ι, κ, η and yeast polymerase ζ were obtained from Enzymax (Lexington, KY). COS-7 cells were purchased from American Type Culture Collection (Rockville, MD). A control undamaged oligodeoxynucleotide (32-mer) with a dC in place of BD N3-dU, was purchased from Midland Reagent Co. (Midland, TX).

Preparation of the nuclear extract. Nuclear extracts were prepared as follows. COS-7 cells were grown in Dulbecco's Modified Eagle Medium and harvested by trypsinization when the cells reached 90% confluence in a T-75 flask. Cells were resuspended in 2 ml of phosphate buffered saline and transferred into microfuge tubes. After concentrating the cells by centrifugation, they were resuspended in 1 ml of cold buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenyl-methylsulfonyl fluoride). The cells were then swollen and lysed by placing on ice for 10 min. A total of 25 µl of 10% Nonidet P-40 was added to the cells and the tubes inverted 10 times to mix the contents. The contents were centrifuged for 30 sec at 4°C at 13,200 rpm and the supernatant discarded. Cold buffer A (1 ml) was added to the pellet and it was resuspended and spun again for 30 sec. The supernatant was discarded and an equal volume of buffer C (10 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.1 mM phenyl-methylsulfonyl fluoride) was added to the pellet. Following resuspension, the sample was placed on ice for 15 min. The contents were then spun at 4°C at 6500 rpm for 5 min and the supernatant collected as the nuclear extract. The protein concentration of the nuclear extract was determined using the Bradford assay with BSA as a standard.

DNA substrates - *In vitro* polymerase reactions were carried out using a series of oligodeoxynucleotide primers (14-, 15- or 16-mers) annealed to templates with the following sequence, 5′-ACCATGCCTGCAAGAA**U***TAAGCAATGATCGCC-3′, where **U*** represents the site of the BD N3-dU adducts or control dC. Primer oligodeoxynucleotides were first phosphorylated with T4 polynucleotide kinase using [γ-^{32}P] ATP and purified using P-6 Bio-Spin columns supplied with Tris-HCl buffer (pH 7.4). The ^{32}P-labeled primers were then mixed with either adducted or non-adducted template oligodeoxynucleotides at a molar ratio of 1:4 in 25 mM Tris HCl buffer (pH 7.6), 50 mM NaCl, heated at 90°C for 3 min, and cooled to RT overnight. To confirm the completion of primer annealing, aliquots were assayed on a 7.5% native PAGE or digested with the restriction enzyme, DpnII that recognizes the restriction site GATC and the products were analyzed on a 20% denaturing polyacrylamide gel.

DNA polymerase assays. *In vitro* polymerization assays were carried out using two polymerase buffers: Pol A buffer containing 25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.1mg/ml BSA, and 10% glycerol and Pol B buffer containing 25 mM Tris-HCl (pH 7.5), 10 mM NaCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mg/ml BSA, and 10% glycerol.

For the primer extension reactions catalyzed by polymerases ι, κ, and η, the reaction mixtures contained 5 nM 14-mer primer/template DNA in Pol A buffer and dNTPs at a final concentration of 10 μM. Reactions were incubated for increasing time intervals (0, 0.5, 1, 2, 5, 10, 15 min) at RT with 10 nM pol ι, 5 nM pol κ and 5 nM pol η. The pol ζ reaction mixture contained 5 nM 14-mer primer/template DNA in Pol B buffer

and dNTPs at a final concentration of 100 μM and were incubated for increasing time intervals (0, 0.5, 1, 2, 5, 10, 15 min) at RT with 14 nM of the polymerase.

For the single nucleotide incorporation reactions, the pol η reaction mixture contained 5 nM 15-mer primer/template DNA in Pol A buffer and each of the four dNTPs at a final concentration of 10 μM and was incubated for 10 min at RT with 0.5 nM of the polymerase. The pol ι reaction mixture contained 5nM 15-mer primer/template DNA in Pol A buffer and each of the four dNTPs at a final concentration of 10 μM and were incubated for 10 and 20 min at RT with 1 nM of the polymerase. The pol κ reaction mixture contained 5nM 15-mer primer/template DNA in Pol A buffer and each of the four dNTPs at a final concentration of 10 μM and were incubated for 10 and 40 min at RT with 2.6 nM of the polymerase. The pol ζ reaction mixture contained 5nM 15-mer primer/template DNA in Pol B buffer and each of the four dNTPs at a final concentration of 10 μM and were incubated for 10 and 60 min at RT with 3.5 nM of the polymerase.

For the extension assays using pol η, the reaction mixture contained 5 nM 16-mer primer/template DNA in Pol A buffer and dNTPs at a final concentration of 100 μM and were incubated for increasing time intervals (0, 1, 3, 5, 7 min) at RT with 1 nM of the polymerase. These experiments were repeated in triplicate. The percentage of extension was quantitated as the (intensity of the bands from the +2 position)/ (total intensity of the bands) and then plotted against time. Quantitation from the +2 position was selected due to a pause site at the +1 position. The average rates of the reactions were then determined from the slopes of the polymerization reactions. The fold difference was calculated as the ratio of (rate of extension for control)/ (rate of extension for the adduct).

For the heparin trapping experiments with pol η, the reaction mixture contained 5 nM 14-mer or 15-mer primer/template DNA in Pol A buffer without $MgCl_2$ and with dNTPs at a final concentration of 10 μM and was preincubated with 2.5 nM of the polymerase for 2 min at RT. The reaction was initiated by addition of $MgCl_2$ or $MgCl_2$ and heparin at a final concentration of 5 mM $MgCl_2$ and 100 μg/ml heparin and incubated for 5 min at RT. To test the effectiveness of the trap, the polymerase was preincubated with $MgCl_2$ and heparin for 7 min at RT and then added to the reaction mixture containing the primer/template DNA and dNTPs at a final concentration of 10 μM and incubated for another 5 min at RT.

For the extension assays using yeast pol ζ, the reaction mixture contained 5 nM 16-mer primer/template DNA in Pol B buffer and dNTPs at a final concentration of 100 μM and was incubated for increasing time intervals (0, 1, 3, 5, 7 min) at RT with 10 nM of the polymerase. These experiments were repeated in triplicate. The percentage of extension was quantitated as the (intensity of the bands from the +2 position)/ (total intensity of the bands) and then plotted against time. Quantitation from the +2 position was selected due to a pause site at the +1 position. The average rates of the reactions were then determined from the slopes of the polymerization reactions. The fold difference was calculated as the ratio of (rate of extension for control)/ (rate of extension for the adduct).

The combined effect of yeast pol δ and ζ to bypass the BD N3-dU adducts was tested. The yeast pol δ reaction mixture contained 5 nM 14-mer primer/template in buffer containing 40 mM Tris HCl (pH 7.8), 0.2 mg/ml BSA, 8 mM Mg Acetate, 0.05 mM ATP with dNTPs at a final concentration of 100 μM. The reaction was incubated for 15 min at RT with 0.8 nM of the polymerase. The pol ζ reaction mixture contained 5 nM

14-mer primer/template in buffer containing 40 mM Tris HCl (pH 7.8), 0.2 mg/ml BSA, 8 mM Mg acetate, 0.05 mM ATP with dNTPs at a final concentration of 100 μM. The reaction was incubated for 15 min at RT with 7 nM of the polymerase. The combined reaction mixture contained 5 nM 14-mer primer/template in buffer containing 40 mM Tris HCl (pH 7.8), 0.2 mg/ml BSA, 8 mM Mg acetate, 0.05 mM ATP and dNTPs at a final concentration of 100 μM. The mixture was incubated for 15 min at RT with 0.8 nM of yeast pol δ and 7 nM of pol ζ.

In assays designed to test for a synergistic reaction between pol η and pol ζ, the pol η reaction mixture contained 5 nM 15-mer primer/template in Pol B buffer with dNTPs at a final concentration of 10 μM. The reactions were incubated for 20 min at RT with 1 nM of the polymerase. The pol ζ reaction mixture contained 5 nM 15-mer primer/template in Pol B buffer with dNTPs at a final concentration of 10 μM. The reaction was incubated for 20 min at RT with 20 nM of the polymerase. The combined reaction mixture contained 5 nM 15-mer primer/template in Pol B buffer and dNTPs at a final concentration of 10 μM. The mixture was incubated for 20 min at RT with 1 nM of pol η and 20 nM of pol ζ. Following termination of these reactions, lanes 1 and 5 were loaded with 2.5 μl of the pol η reaction mixture for the control and adducts, respectively; lane 2 and 6 were loaded with 2.5 ul of the pol ζ reaction mixture for the control and adducts, respectively; lanes 3 and 7 were loaded with 2.5 μl aliquots from the individual reactions of pol η and pol ζ for the control and adducts, respectively following termination of those reactions. Lanes 4 and 8 were loaded with 5 μl of the concomitant reaction mixture for the control and adducts, respectively. These experiments were performed in duplicate. The DNA products of the polymerization were quantitated by

calculating the extension as the (intensity of the bands from the +1 position)/ (total intensity of the bands) for the additive lanes 3 and 7 and the concomitant lanes 4 and 8. The extension for the concomitant lane was divided by the extension for the additive lane to give a ratio that signifies the synergistic effect.

All reactions were terminated by the addition of stop solution consisting of 95% (v/v) formamide, 20 mM EDTA, 0.02% (w/v) xylene cyanol, and 0.02% (w/v) bromphenol blue. Reaction products were resolved on a 15% denaturing gel containing 8 M urea and visualized by autoradiography. Quantitative analyses were performed using a PhosphorImager screen and Image-Quant software.

RESULTS

Survey of translesional polymerases on the BD N3-dU adducted template. In the previous chapter we determined that the DNA replicative polymerases pol δ and ε were blocked one nucleotide prior to BD N3-dU adducts. Since replication through COS-7 cells of plasmids containing these lesions yielded a high mutagenic frequency, we initiated an investigation of whether specific translesion polymerases or a combination of polymerases, could bypass the BD N3-dU adducts. Thus, polymerases ι, κ, η and ζ were examined. In the case of pol ι, while extension was observed with the control unadducted DNA, primer extension using a template containing the BD N3-dU adducts was significantly blocked one nucleotide 3′ of the lesion (Fig. 3.2A), although a minor amount of bypass was observed. Qualitatively similar results were observed using pol κ, such that the adducts were highly blocking to the polymerase at the –1 position and bypass synthesis was minimal (Fig. 3.2B). In contrast, primer extension reactions using pol η, resulted in a significant incorporation of nucleotides opposite the adducts (Fig. 3.2C). Formation of full-length product was also seen with pol η. However, the majority of the synthesis was blocked following incorporation of nucleotides opposite the lesion (0 position). When the primer extension reactions were carried out with pol ζ, significant blockage was seen 1 nucleotide prior to the site of the adducts (Fig. 3.2D). However, the limited amount of incorporation opposite the adducts also led to extension to full- length product, suggesting that pol ζ could extend a primer when the incorporation step opposite the lesion was already completed. Thus pol η could serve as an incorporator and pol ζ could serve as an extendor past the BD N3-dU adducts.

5'GGCGATCATTGCTT3'
3'CCGCTAGTAACGAATU*AAGAACGTCCGTACCA5'

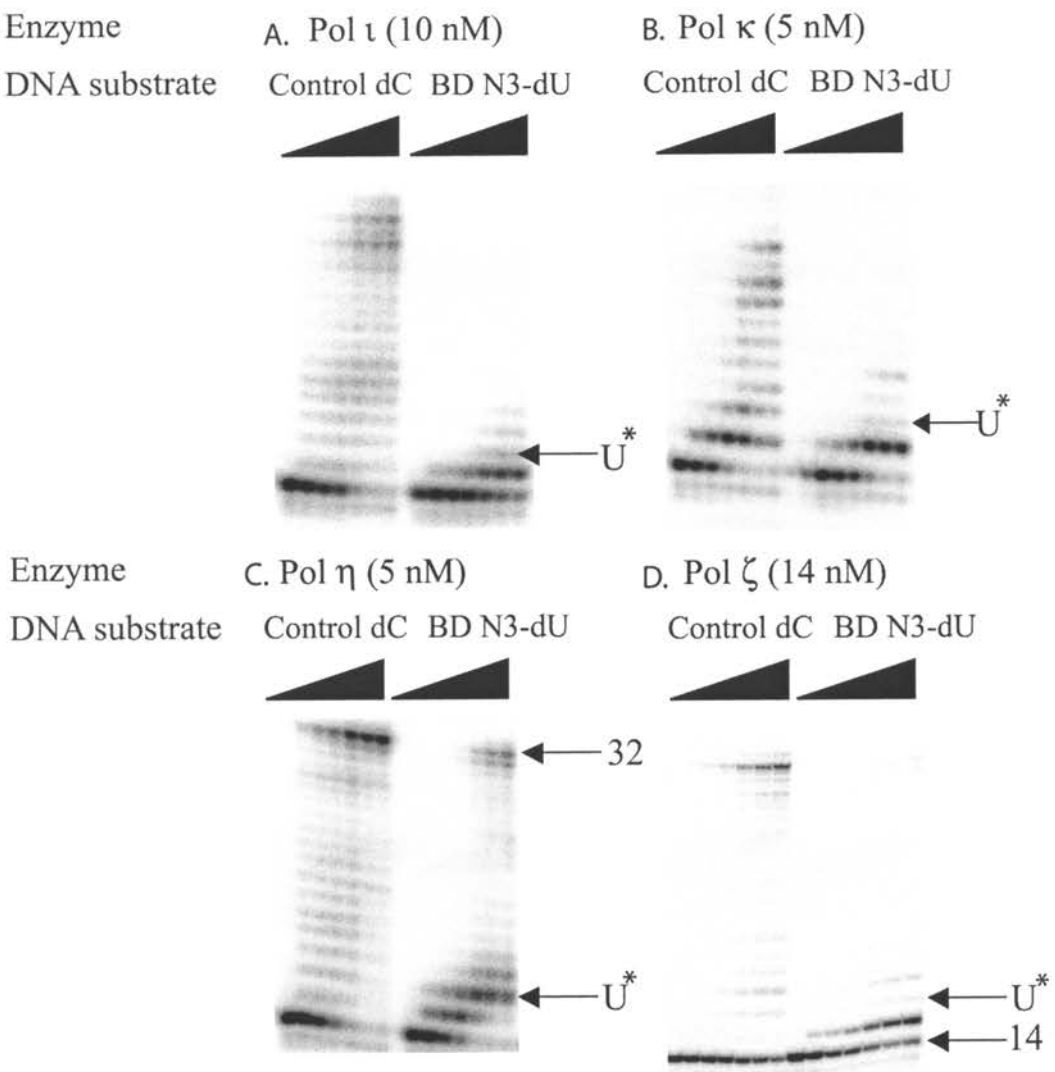


Fig. 3.2. Primer extension reactions catalyzed by polymerases ι, κ, η or ζ on the BD N3-dU adducted template. Control dC or BD N3-dU adducted 32-mer DNA templates

were annealed to a -2 primer. Time course experiments (at time intervals 0, 0.5, 1, 2, 5, 10 and 15 min) were carried out with the DNA substrates (5 nM) at RT in the presence of all four dNTPs (10 μM in the case of ι, κ, η and 100 μM in the case of pol ζ) with the indicated concentration of polymerases ι, κ, η or ζ. The positions of the 14-nucleotide primers and the 32-nucleotide full-length products are indicated. U* indicates the position of the modified U on the template.

Examination of replication past the BD N3-dU adducted template with a mammalian nuclear extract. Having surveyed four translesional polymerases for bypass of BD N3-dU containing DNA and found that only pol η could incorporate nucleotides opposite the adducts, we wanted to test whether another polymerase within the cell might catalyze this reaction more efficiently. To globally survey for such an activity, a nuclear extract from COS-7 cells was prepared and the ability to replicate past the BD N3-dU adducts was tested. As seen in Fig. 3.3, extended kinetic analysis showed that there was a near total blockage to replication at the –1 position. The integrity of the polymerase preparation was tested by the replication of control DNA. These data reveal that the major polymerases in the nuclear extract were not processive even on the control substrate, although the preparation was able to carry out DNA synthesis. These data suggest that there is not a highly efficient bypass polymerase for replication of the BD N3-dU adducts present in the nuclear extract.

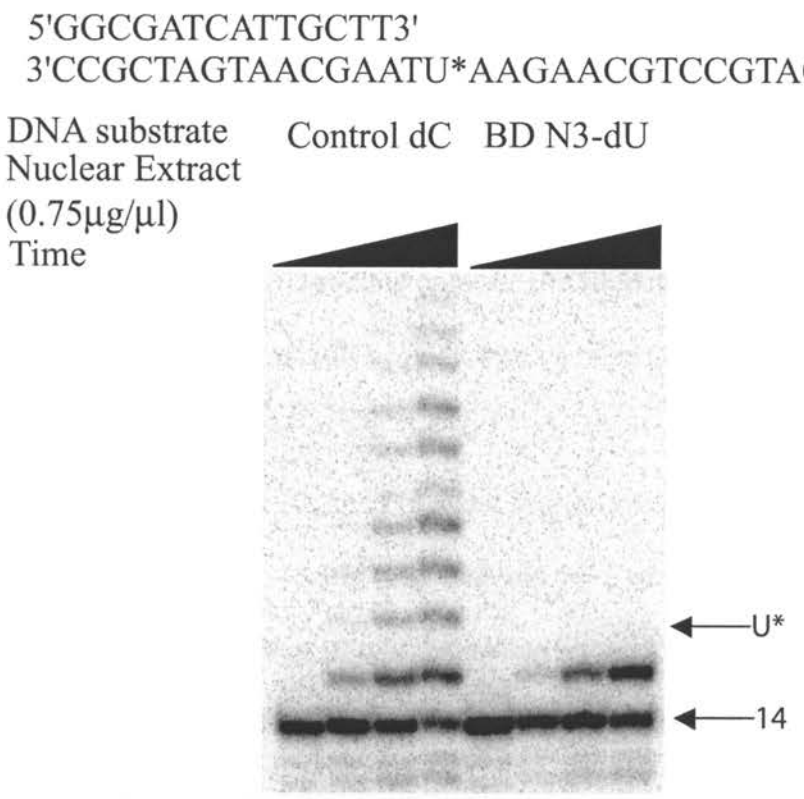


Fig. 3.3. Examination of replication past the BD N3-dU adducted template with a mammalian nuclear extract. Control dC or BD N3-dU adducted 32-mer DNA templates were annealed to a -2 primer. Kinetic experiments (at time intervals of 0, 10, 15 and 30 min) were carried out with the DNA substrates (5 nM) at RT in the presence of all four dNTPs (100 μM) with 0.75 μg/μl of the nuclear extract in the reaction mixture. The position of the 14-mer nucleotide primer is indicated. U* indicates the position of the modified U on the template.

Single nucleotide incorporation assays with pol η, pol ι, pol κ, and pol ζ. Based on the data presented in Fig. 3.2 and 3.3, I determined the specificity of nucleotide incorporation by pol η opposite the BD N3-dU adducts. As seen in Fig. 3.4, pol η was able to incorporate all four nucleotides opposite the adducts with a preference of G and A. I had previously shown in a cellular mutagenesis assay (Fernandes et al., 2006) that A and T were the preferential nucleotides incorporated opposite the adducts. In addition to pol η, the specificity of nucleotide incorporation by pol ι, κ and ζ opposite the BD N3-dU adducts was also determined. As seen in Fig. 3.5, pol ι was able to incorporate G and T nucleotides opposite the adducts in 20 min. Pol κ was able to incorporate C and T nucleotides opposite the adducts in 40 min (Fig. 3.6) and pol ζ was unable to incorporate any nucleotide opposite the adducts in 10 min, indicating how poor it was at the incorporation step. However, in 60 min it favored the incorporation of T nucleotide opposite the adduct (Fig. 3.7). These data collectively suggest that pol η may be one of the translesional polymerases responsible for synthesis opposite the BD N3-dU adducts.

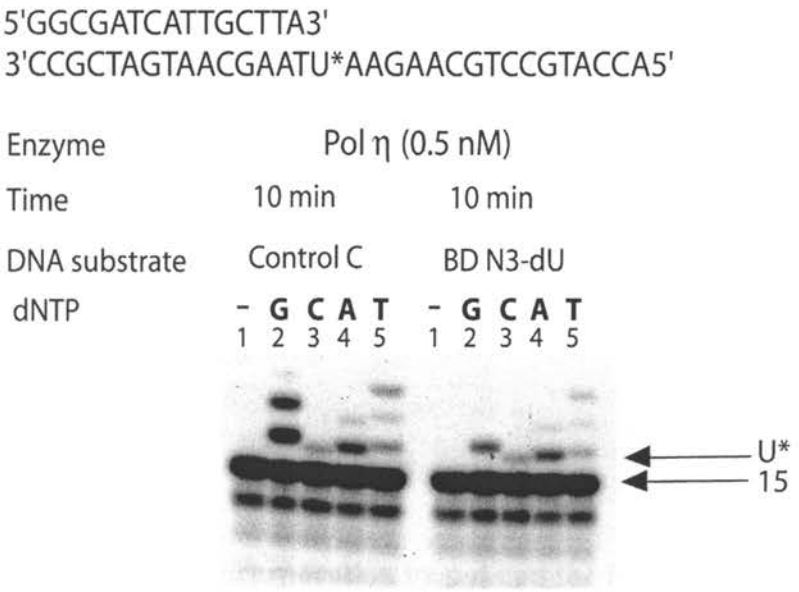


Fig. 3.4. Single-nucleotide incorporation by pol η on the BD N3-dU adducted template. Control dC or BD N3-dU adducted 32-mer DNA templates were annealed to a - 1 primer. The DNA substrates (5 nM) were incubated at RT for 10 min with 0.5 nM pol η in the presence of 10 μM of each of the four dNTPs. The position of the 15-nucleotide primer is indicated. U* indicates the position of the modified U on the template.

5'GGCGATCATTGCTTA3'
3'CCGCTAGTAACGAATU*AAGAACGTCCGTACCA5'

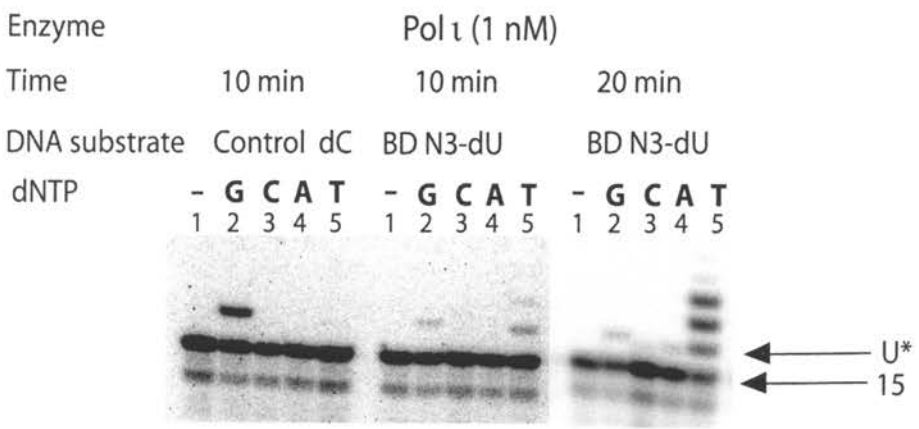


Fig. 3.5. Single-nucleotide incorporation by pol ι on the BD N3-dU adducted template. Control dC or BD N3-dU adducted 32-mer DNA templates were annealed to a - 1 primer. The DNA substrates (5 nM) were incubated at RT for 10 or 20 min with 1 nM pol ι in the presence of 10 μM of each of the four dNTPs. The position of the 15-nucleotide primer is indicated. U* indicates the position of the modified U on the template.

5'GGCGATCATTGCTTA3'
3'CCGCTAGTAACGAATU*AAGAACGTCCGTACCA5'

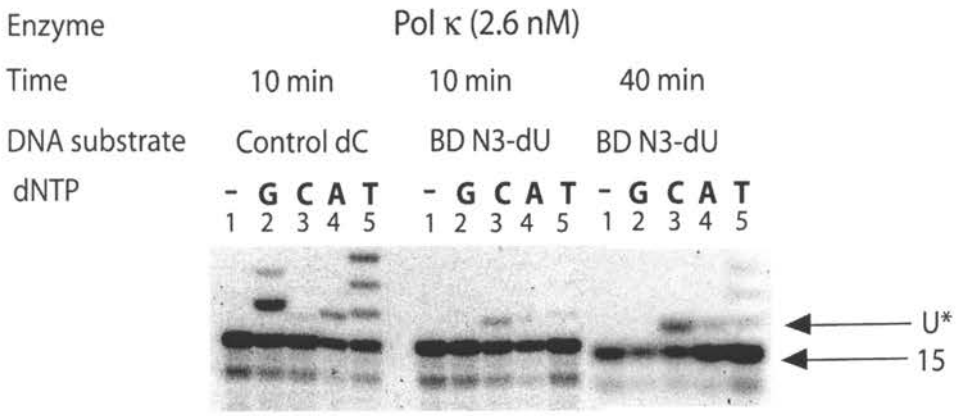


Fig. 3.6. Single-nucleotide incorporation by pol κ on the BD N3-dU adducted template. Control dC or BD N3-dU adducted 32-mer DNA templates were annealed to a - 1 primer. The DNA substrates (5 nM) were incubated at RT for 10 or 40 min with 2.6 nM pol κ in the presence of 10 μM of each of the four dNTPs. The position of the 15-nucleotide primer is indicated. U* indicates the position of the modified U on the template.

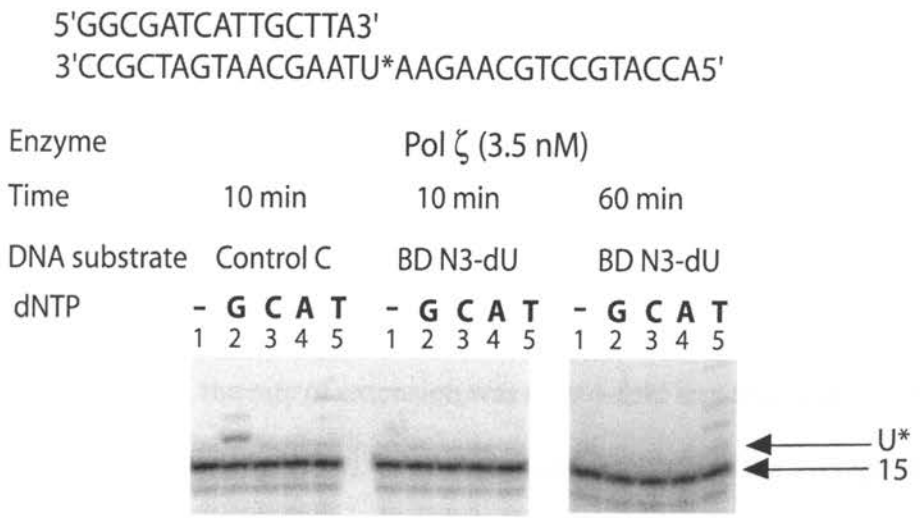


Fig. 3.7. Single-nucleotide incorporation by pol ζ on the BD N3-dU adducted template. Control dC or BD N3-dU adducted 32-mer DNA templates were annealed to a - 1 primer. The DNA substrates (5 nM) were incubated at RT for 10 or 60 min with 3.5 nM pol ζ in the presence of 10 μM of each of the four dNTPs. The position of the 15-nucleotide primer is indicated. U* indicates the position of the modified U on the template.

Primer extension reactions catalyzed by pol η past the BD N3-dU adducts annealed to different mismatched primers. Since formation of full-length product was seen with pol η, the ability of pol η to catalyze extension of different mismatched 3′ termini that were positioned opposite the BD N3-dU adducts was examined. Fig. 3.8 and Table 3.1 show the kinetic analyses of extension past the adducts. When G was placed opposite the adducts (16G/adduct), the rate of extension was only 4-fold less than that of the correctly matched control primer (Fig. 3.8A). In all four cases, analyses of the average rates of extension from three independent experiments revealed that there were only modest reductions in the rates of extension of the primer hybridized to the adduct containing template relative to that of the corresponding control (Table 3.1). These data suggest that pol η can extend a mismatched terminus irrespective of whether there is a lesion or not. The fold difference between the correctly paired primer template and the 16T/adducted template was the least, indicating that pol η favors best the extension of T opposite the lesion (Fig. 3.8D), followed by extension of A opposite the lesion (Fig. 3.8C). The fold difference between correctly paired primer template and extension of a C opposite the adducted template (16C/adduct) was the greatest, indicating that pol η does not favor extension of C opposite these lesions (Fig. 3.8B). Thus pol η can most efficiently extend the A and T mismatches opposite the adducts.

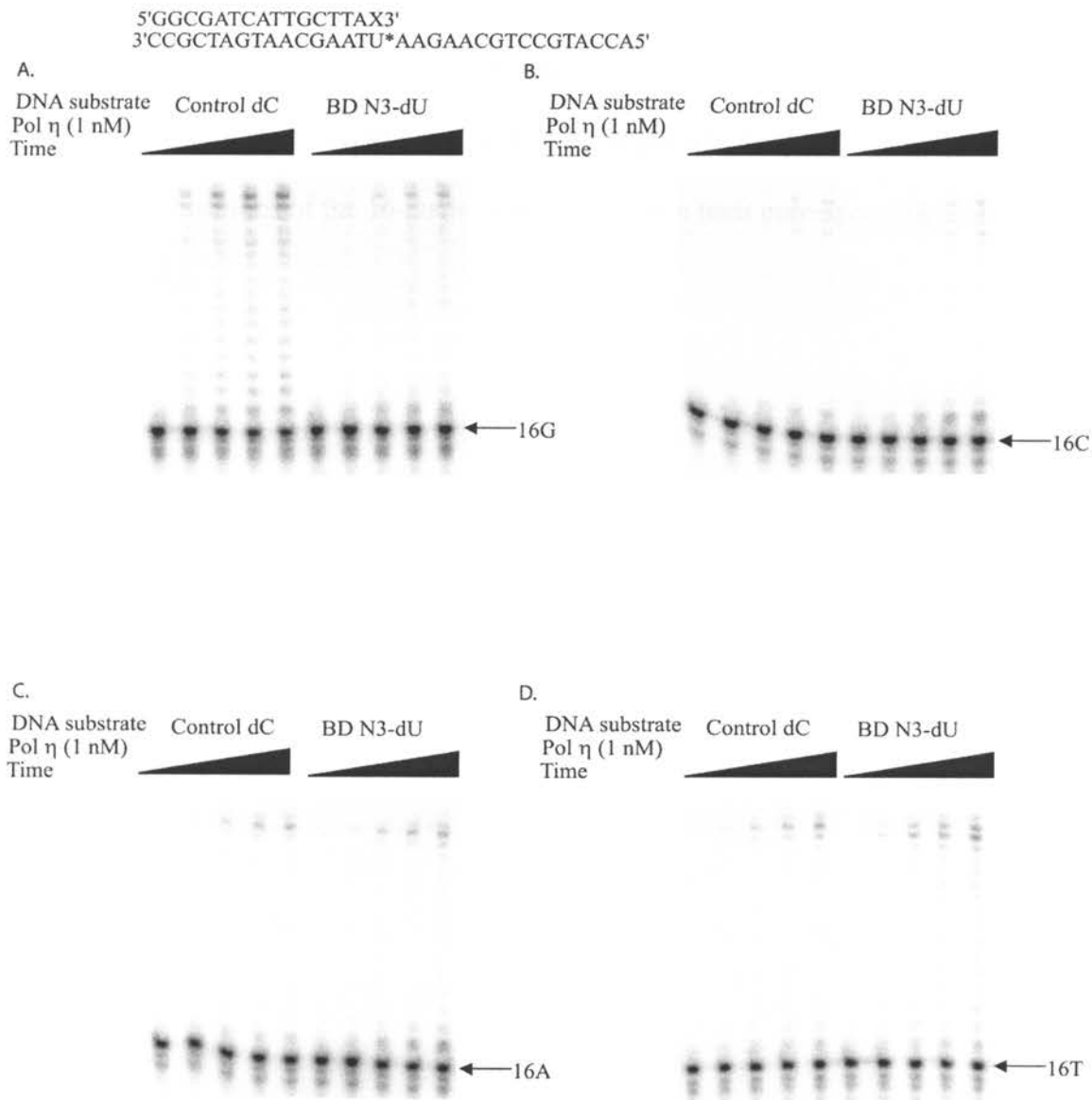


Fig. 3.8. Primer extension reactions catalyzed by pol η past the BD N3-dU adducts annealed to different mismatched primers. X in the DNA sequence corresponds to G, C, A or T. (A) Control dC or BD N3-dU 32-mer DNA templates were annealed to a 0 primer with G opposite the control dC or BD N3-dU adducts. (B) Control dC or BD N3-dU 32-mer DNA templates were annealed to a 0 primer with C opposite the control dC or BD N3-dU adducts. (C) Control dC or BD N3-dU 32-mer DNA templates were annealed

to a 0 primer with A opposite the control dC or BD N3-dU adducts. (D) Control dC or BD N3-dU 32-mer DNA templates were annealed to a 0 primer with T opposite the control dC or BD N3-dU adducts. The DNA substrates (5nM) were incubated at RT for time intervals of 0, 1, 3, 5, and 7 min with 1 nM of pol η in the presence of 100 μM dNTPs. The positions of the 16-nucleotide primers with their corresponding mismatches are indicated.

Substrate	Average rate of extension	Standard deviation	Fold difference over corresponding control primer	Fold difference over correctly matched control primer
16 G/Control	5.5	0.16	4.01	4.01
16 G/Adduct	1.37	0.17		
16 C/Control	0.48	0.13	0.46	5.2
16 C/Adduct	1.04	0.12		
16 A/Control	1.4	0.23	0.6	2.4
16 A/Adduct	2.3	0.19		
16 T/Control	1.94	0.21	0.43	1.2
16 T/Adduct	4.44	0.89		

Table 3.1. Extension past the BD N3-dU containing templates annealed to mismatched primers by pol η

Heparin trapping of pol η. In order to determine if the incorporation and extension past the BD N3-dU adducts involves a single or multiple encounters with the polymerase, trapping reactions of the polymerase were carried out using heparin. In the presence of heparin, the 14-mer primer DNA (Substrate A) was extended by only 1 nucleotide (Fig. 3.9, lane 4), with no observation of insertion opposite the adducts. Thus, these data indicate that incorporation and extension by pol η past the BD N3-dU adducts requires more than one encounter by the polymerase. In the case of the 15-mer primer DNA (Substrate B), and in the presence of heparin, there was no incorporation or extension past the adducts (Fig. 3.9, lane 8), again indicating that more than one encounter with the polymerase was needed to generate a detectable product.

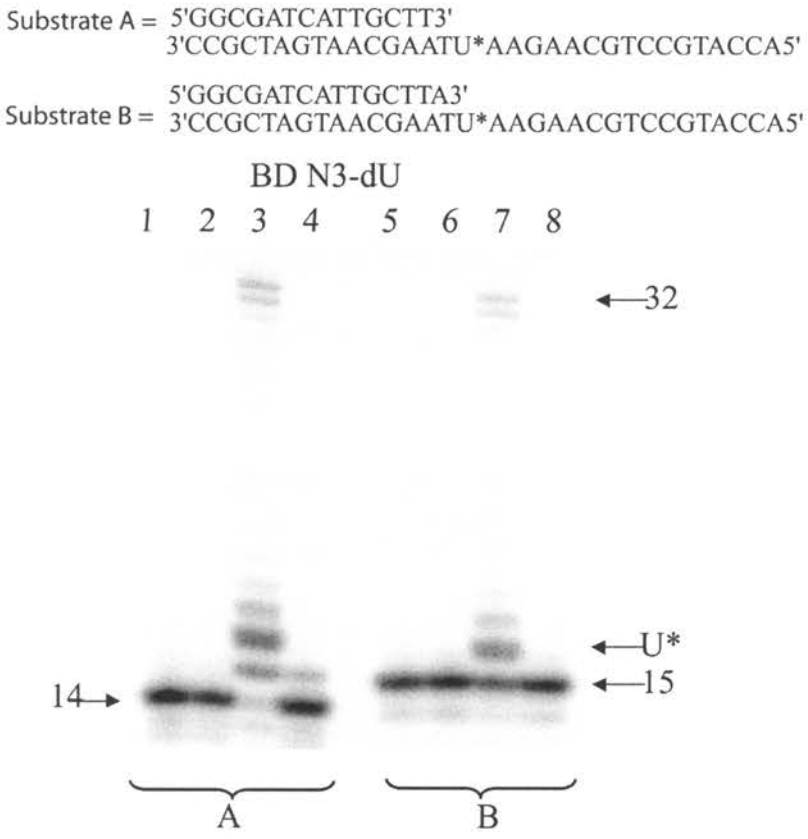


Fig. 3.9. Heparin trapping of pol η. BD N3-dU adducted templates were annealed to a –2 (Substrate A) or -1 primer (Substrate B). Lanes 1 and 5 represent DNA substrates (5 nM) A and B respectively. Lane 2 and 6 represent reactions in which pol η is preincubated with heparin and $MgCl_2$ for 7 min at RT and then added to the DNA substrates (5 nM) in the presence of 10 μM dNTPs. Lanes 3 and 7 represent reactions in which DNA substrates (5 nM) were incubated for 5 mins at RT with 2.5 nM of pol η in the presence of 10 μM dNTPs and 5 mM $MgCl_2$. Lanes 4 and 8 represent reactions in which DNA substrates (5 nM) were incubated for 5 mins at RT with 2.5 nM of pol η in the presence of 10 μM dNTPs, 5 mM $MgCl_2$ and 100 μg/ml heparin. The positions of the

14-nucleotide and 15-nucleotide primers and the 32-nucleotide full-length products are indicated. U* indicates the position of the modified U on the template.

Primer extension reactions catalyzed by pol ζ past the BD N3-dU adducts annealed to different mismatched primers. Since pol ζ is known to be an extender of mismatched termini (Johnson et al., 2000b), its ability to catalyze extension of different mismatched 3′ termini that were positioned opposite the BD N3-dU adducts was examined. Fig. 3.10 and Table 3.1 show the kinetic analyses of extension past the adducts. When G was placed opposite the adducts (16G/adduct), the rate of extension was only 4.13-fold less than that of the correctly matched control primer (Fig. 3.10A). In all four cases, analyses of the average rates of extension from three independent experiments revealed that there were only modest reductions in the rates of extension of the primer hybridized to the adduct containing template relative to that of the corresponding control (Table 3.2). These data suggest that pol ζ can extend a mismatched terminus irrespective of whether there is a lesion or not. The fold difference between the correctly paired primer template and the 16A/adducted template was the least indicating that pol ζ favors the extension of A opposite the lesion (Fig. 3.10C). The fold difference between correctly paired primer template and extension of a C opposite the adducted template (16C/adduct) was the greatest, indicating that pol ζ does not favor extension of C opposite these lesions (Fig. 3.7B). In the case when T was placed opposite the adducts (16T/adduct), two extension products were seen (Fig. 3.10D). This may be due to a slippage mechanism of the polymerase, whereby it translocates to the next templating nucleotide and forms a product that is one nucleotide shorter than the full-length product. Therefore pol ζ can extend most efficiently from an A opposite the adduct.

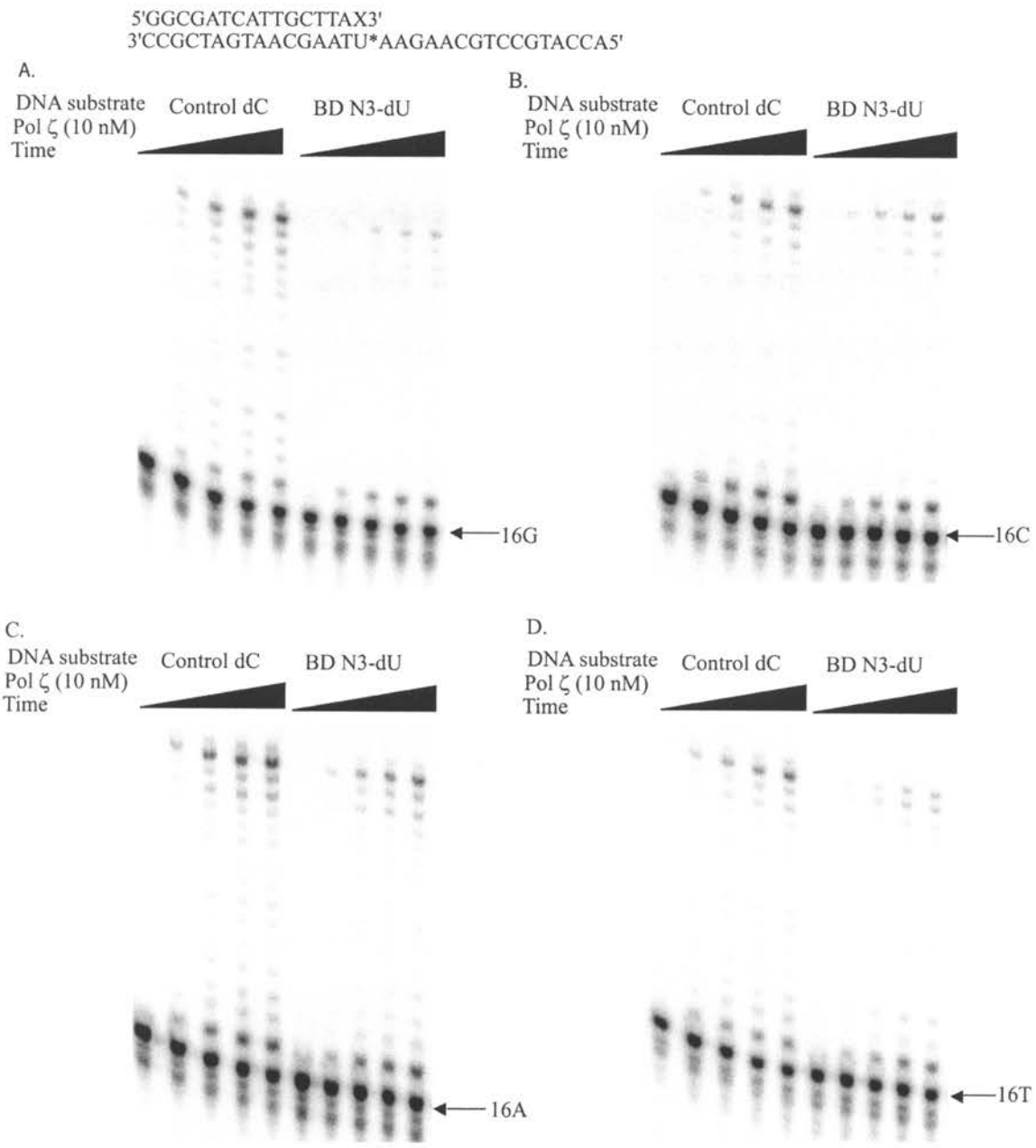


Fig. 3.10. Primer extension reactions catalyzed by pol ζ past the BD N3-dU adducts annealed to different mismatched primers. X in the DNA sequence corresponds to G, C, A or T. (A) Control dC or BD N3-dU 32-mer DNA templates were annealed to a 0 primer with G opposite the control dC or BD N3-dU adducts. (B) Control dC or BD N3-dU 32-mer DNA templates were annealed to a 0 primer with C opposite the control dC or BD N3-dU adducts. (C) Control dC or BD N3-dU 32-mer DNA templates were annealed

to a 0 primer with A opposite the control dC or BD N3-dU adducts. (D) Control dC or BD N3-dU 32-mer DNA templates were annealed to a 0 primer with T opposite the control dC or BD N3-dU adducts. The DNA substrates (5nM) were incubated at RT for time intervals of 0, 1, 3, 5, and 7 min with 10 nM of pol ζ in the presence of 100 μM dNTPs. The positions of the 16-nucleotide primers with their corresponding mismatches are indicated.

Substrate	Average rate of extension	Standard deviation	Fold difference over corresponding control primer	Fold difference over correctly matched control primer
16 G/Control	5.49	1.26	4.13	4.13
16 G/Adduct	1.33	0.66		
16 C/Control	1.3	0.81	1.38	5.81
16 C/Adduct	0.94	0.16		
16 A/Control	2.95	0.69	1.52	2.82
16 A/Adduct	1.93	0.37		
16 T/Control	4.28	0.39	2.94	3.77
16 T/Adduct	1.46	0.36		

Table 3.2. Extension past the BD N3-dU containing templates annealed to mismatched primers by pol ζ

Examination of replication past the BD N3-dU adducted template with yeast pol δ and pol ζ. Although yeast pol δ was blocked by the BD N3-dU adducts, mammalian pol δ was able to bypass the BD N3-dU adducts at high concentrations (Fernandes et al., 2006). We therefore wanted to test the effect of yeast pol δ in combination with pol ζ. As seen in Fig. 3.11, the combined action of yeast pol δ and ζ did not yield any more extension past the adducts than that of the individual polymerases, thus indicating that these polymerases in combination may not promote the bypass of these adducts *in vivo*.

5'GGCGATCATTGCTT3'
3'CCGCTAGTAACGAATU*AAGAACGTCCGTACCA5'

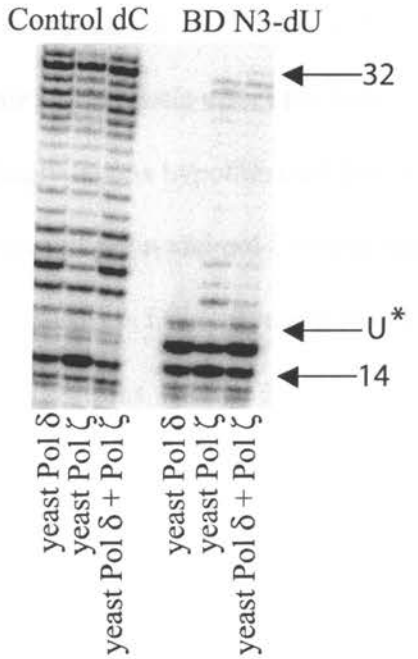


Fig. 3.11. Examination of replication past the BD N3-dU adducted template with yeast pol δ and pol ζ. Control dC or BD N3-dU adducted templates were annealed to a –2 primer. The DNA substrates (5nM) were incubated for 15 mins at RT with either 0.8 nM of yeast pol δ, 7 nM of pol ζ or a combination of 0.8 nM yeast pol δ and 7 nM pol ζ in the presence of 100 μM dNTPs. The positions of the 14-nucleotide primers and the 32-nucleotide full-length products are indicated. U* indicates the position of the modified U on the template.

Synergistic Effect between pol η and pol ζ in the bypass of the BD N3-dU adducts.

Since pol η and pol ζ were identified as the most efficient enzymes to catalyze nucleotide incorporation opposite and extension beyond the BD N3-dU adducts, respectively, experiments were designed to test for a synergistic effect for both these polymerases in the bypass of these lesions. Accordingly, it was hypothesized that polymerization reactions using a simultaneous addition of pol η and pol ζ would result in the accumulation of significantly more full-length DNA products than if the pol η and pol ζ reactions were carried out in separate reactions. In Fig. 3.12, lanes 1 and 5 show the DNA products of a pol η reaction using control and adduct-containing oligodeoxynucleotides, respectively, while lanes 2 and 6 show the same reactions, except using pol ζ. The DNAs in lanes 3 and 7 represent the additive polymerization reaction, in which aliquots of the individual pol η and pol ζ reactions (lanes 1 and 2) and (lanes 5 and 6) were physically combined following the termination of the reaction for the control and adduct-containing samples, respectively. Lanes 4 and 8 represent the concomitant polymerization reaction of pol η and pol ζ on the control and adduct-containing oligodeoxynucleotides, respectively. These experiments were performed in duplicate and the average ratio of concomitant effect / additive effect was determined. This ratio was found to be 1.18 for the control and 2.7 for the adduct-containing DNA, thus indicating that there was a significant synergy in the production of full-length DNA products on the adduct-containing substrate when the two polymerases were simultaneously added. The concomitant reaction of pol η and pol ζ also showed the incorporation of two different nucleotides opposite the adducts, yielding data that are consistent with the results from

the single nucleotide incorporation assay for pol η. Therefore these data indicate a synergistic action between pol η and pol ζ to catalyze bypass of these adducts.

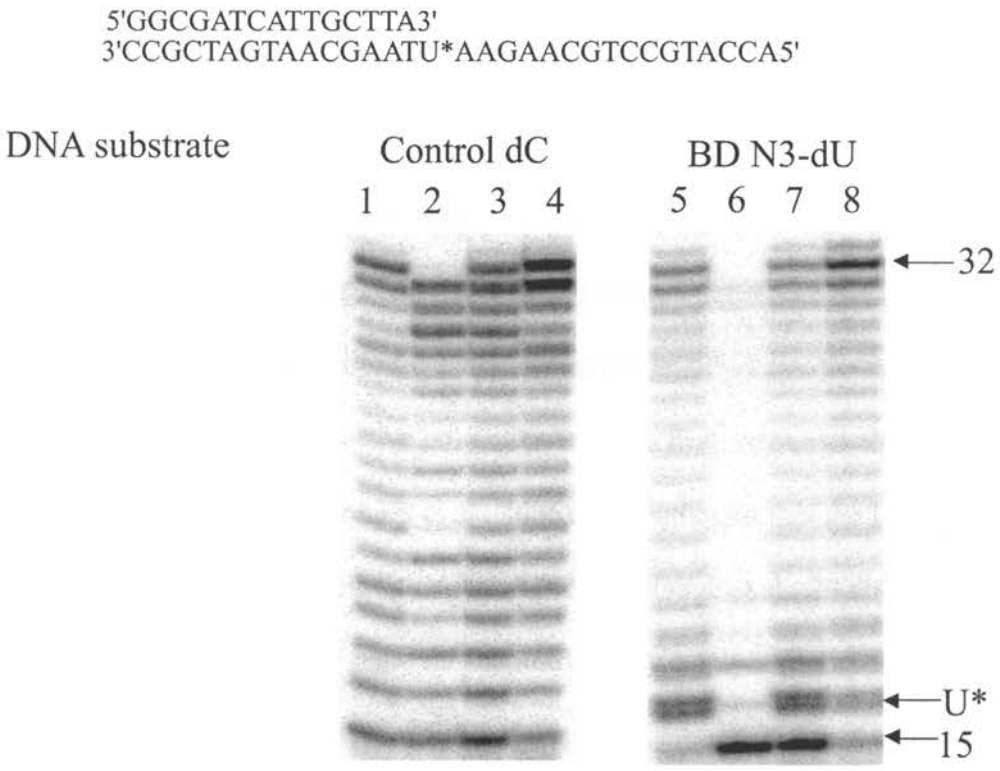


Fig. 3.12. Synergistic effect of pol η and pol ζ on the BD N3-dU adducted template. Control dC or BD N3-dU adducted templates were annealed to a –1 primer. Lanes 1 and 5 represent 2.5 μl of the DNA substrates (5 nM) that were incubated at RT for 20 min with 1 nM of pol η in the presence of 10 μM dNTPs. Lanes 2 and 6 represent 2.5 μl of the *DNA substrates (5 nM) that were incubated at RT for 20 min with 20 nM of pol ζ in the* presence of 10 μM dNTPs. Lanes 3 and 7 indicate 2.5 μl aliquots from the individual reactions of pol η (Lane 1 and 5) and pol ζ (Lane 2 and 6) after termination of those reactions. Lane 4 and 8 represent 5 μl of the DNA substrates (5 nM) that were incubated at RT for 20 min with 1 nM of pol η and 20 nM of pol ζ. The positions of the 15-nucleotide primers and the 32-nucleotide full-length products are indicated. U* indicates the position of the modified U on the template.

DISCUSSION

Data presented within this study reveal that the BD N3-dU adducts are highly blocking lesions. However, they can be bypassed and extended by pol η alone or in combination with pol ζ, with some degree of synergy in the latter case. I propose a model for the bypass of the BD N3-dU adducts (Fig. 3.13) that is based on the two polymerase two-step mechanism of translesion synthesis (Prakash and Prakash, 2002; Woodgate, 2001). In this model, the replicative polymerases δ or ε first encounter the BD N3-dU adducts and are blocked (Fernandes et al., 2006). I hypothesize that the blocked replication fork leads to the ubiquitination of PCNA (Hoege et al., 2002; Kannouche et al., 2004) and a recruitment of pol η that can efficiently catalyze incorporation opposite the adducts, even though this may require multiple enzyme-DNA encounters. Such a role for pol η would further expand the scope of lesions that it can bypass including cyclobutane pyrimidine dimers (Johnson et al., 1999b; Masutani et al., 1999a). Further extension past the adduct was very poor as evidenced by a significant block at the 0 position. However, in separate experimental data generated using primers that were mismatched at the 3′-termini revealed extension that was almost as efficient as control unadducted oligodeoxynucleotides. Although these data may appear inconsistent, I hypothesize that following nucleotide incorporation opposite the BD N3-dU adducts, the structure of the mismatched terminus must undergo a conformational change and enzyme dissociation/reassociation prior to further primer extension. In such a bypass pathway when G or A is preferentially incorporated by pol η opposite the adducts, further extension synthesis could be most efficiently catalyzed by either pol η or pol ζ when A is inserted opposite the adducts. Pol ζ was efficient at extension of the 16/A mismatched

primer terminus, although it was completely inefficient at incorporating nucleotides opposite the adducts. This is in concordance with its ability to extend mispaired termini better than its ability to incorporate opposite a lesion (*reviewed in* Prakash et al., 2005). Thus, it is proposed that bypass of the BD N3-dU lesions appears to occur through multiple encounters with pol η alone or the combined action of pol η and pol ζ. Following dissociation of pol η or pol ζ, pol δ or pol ε could resume replication (Fig. 3.13D).

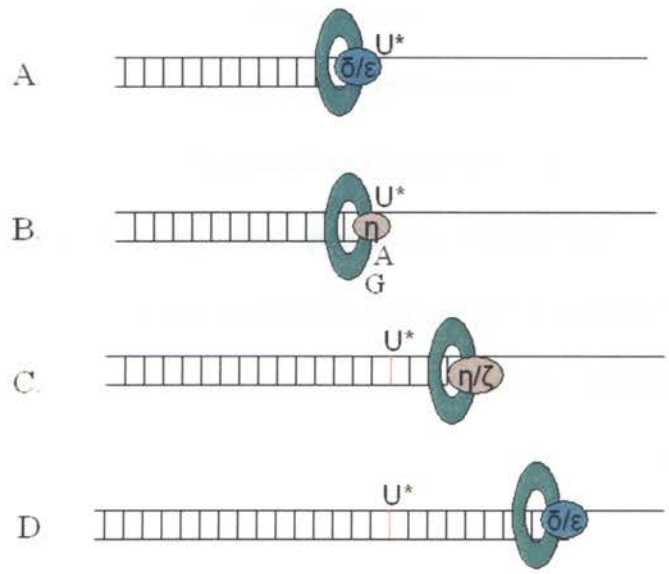


Fig. 3.13. Model for the bypass of the BD N3-dU adducts. (A) Replicative polymerase pol δ or pol ε is blocked by the presence of the BD N3-dU adducts. (B) This leads to ubiquitination of PCNA that subsequently recruits pol η to the site of the stalled replication fork. Pol η is able to incorporate G and A nucleotides opposite the adducts, but further extension is blocked significantly. (C) Another molecule of pol η or pol ζ is capable of removing this block by causing further extension past the adducts when A is inserted opposite them. (D) Once pol ζ or pol η dissociates, replication by pol δ or pol ε resumes.

The data presented on the bypass of the BD N3-dU adducts presented herein agrees well with the types of mutations observed following replication past these lesions in COS-7 cells, which were mainly C to T (53%) and C to A (32.5%) mutations, with a lower number of C to G (11%) mutations (Fernandes et al., 2006). These data suggest that primers with an A incorporated opposite the adducts were well extended during replication in cells, and are in accordance with pol pol η's and pol ζ's ability to extend the A mismatched primer efficiently. Thus overall, these data implicate pol η alone or the combined effect of pol η and pol ζ on the BD N3-dU adducts to be responsible for the induction of C to T mutations in mammalian cells.

To gain a better understanding of the molecular mechanisms underlying butadiene mutagenesis and carcinogenesis, previous investigations have examined the mutagenic potential of a number of BD-derived DNA adducts that have been site-specifically incorporated into vectors and replicated through mammalian or *E. coli* cells (Carmical et al., 2000b; Carmical et al., 2000c; Kanuri et al., 2002b). Of those previously examined only the N1 deoxyinosine adducts were found to be highly mutagenic (Kanuri et al., 2002b). However, this investigation has identified the BD N3-DU adducts as another candidate lesion for high frequency mutagenic bypass. Both adducts are deamination products formed from the reaction of EB with deoxyadenosine or deoxycytosine respectively. It is interesting that although inosine resembles guanine and uracil resembles thymine, the mutations observed with these adducts encompasses all four nucleotides.

In addition to the BD N3-dU adducts, there are other DNA adducts that require the combined action of two or more polymerases. For instance, in the case of 8-oxoG

and m^6G, Pol ζ is inefficient at replicating through these lesions, but can readily extend from them when combined with pol δ (Haracska et al., 2003). In the case of a (6-4) TT photoproduct or an abasic site, pol ζ can carry out extension reactions when Pol ι has inserted a deoxynucleotide opposite these lesions (Johnson et al., 2000b). Efficient bypass of a (6-4) TT PP can also occur through the combined action of pol η and pol ζ, whereby pol η can insert opposite the 3' T of the (6-4) TT PP and pol ζ can extend from it (Johnson et al., 2001). Similarly, Pol ζ can extend from γ-hydroxy-1,N^2-propano-2' deoxyguanosine (γ-HOPdG) adduct when Rev1 efficiently incorporates a C opposite the lesion (Washington et al., 2004b). Another lesion that requires the combined action of pol η, Rev1 and pol ζ are the (+) and (-) *trans-anti*-BPDE-N^2-dG DNA adducts for the induction of G to T mutations in yeast cells (Zhao et al., 2006).

Overall my investigations support complex models for polymerase switching and co-operativity in the bypass of lesions resulting from environmental toxicant exposure and endogenously produced DNA lesions.

ACKNOWLEDGEMENTS

I acknowledge Linda Hackfeld and Dr. Richard Hodge for the synthesis of the BD N3-dU adducted oligodeoxynucleotides and Dr. Irina Minko for guidance on replication analyses.

CHAPTER 4

SITE-SPECIFIC MUTAGENICITY OF STEREOCHEMICALLY DEFINED 1,N^2-DEOXYGUANOSINE ADDUCTS OF *TRANS*-4-HYDROXYNONENAL IN MAMMALIAN CELLS

ABSTRACT

Trans-4-hydroxynonenal (HNE) is a toxic compound produced endogenously during lipid peroxidation. HNE is a potent electrophile that is reactive with both proteins and nucleic acids. HNE preferentially reacts with deoxyguanosine to form four stereoisomeric HNE-deoxyguanosine adducts: *(6R, 8S, 11R), (6S, 8R, 11S), (6R, 8S, 11S),* and *(6S, 8R, 11R)*. These adducts were synthesized into 12-mer oligodeoxynucleotides, inserted into a DNA shuttle vector, and evaluated for the ability of each stereoisomer to induce mutagenesis when replicated through mammalian cells. The resultant mutagenicity of these adducts was related to their stereochemistry, in that two of the HNE-dG adducts, *(6R, 8S, 11R)* and *(6S, 8R, 11S),* were significantly more mutagenic than the *(6R, 8S, 11S)* and *(6S, 8R, 11R)* HNE-dG adducts. These data conclusively demonstrate that HNE-derived DNA adducts are mutagenic in mammalian cells and their potency as a mutagen is dictated by their stereochemistry.

INTRODUCTION

Biological membranes are composed of polyunsaturated fatty acids that are continually exposed to reactive oxygen species, and undergo oxidative degradation resulting in the generation of toxic aldehydic by-products like malonaldehyde, acrolein, crotonaldehyde and *trans*-4-hydroxynonenal (HNE). In the absence of efficient antioxidant defense systems, these products have the potential to mediate various toxic effects (Esterbauer et al., 1991), including genotoxic effects. HNE is mutagenic in Chinese hamster lung cells (Cajelli et al., 1987) and causes G:C to T:A transversions at codon 249 of a wild-type p53 lymphoblastoid cell line (Hussain et al., 2000). It has been suggested that this mutagenic behavior is due to the formation of DNA adducts from reactions with HNE. Racemic HNE can specifically react with deoxyguanosine in DNA, by Michael addition, to yield four diastereomeric propano 1,N^2-deoxyguanosine adducts (Winter et al., 1986). Additionally, Chung et al., (Chung et al., 2000) have reported the presence of these 1,N^2-deoxyguanosine isomeric adducts of HNE as endogenous lesions in rodent and human tissue.

HNE belongs to the group of 4-hydroxyalkenals and is the most representative hydroxyalkenal to be quantitatively detected *in vivo*. HNE is derived from the oxidation of ω-6 polyunsaturated fatty acids, such as arachidonic acid, linoleic acid or their hydroperoxides (Esterbauer et al., 1991; Schneider et al., 2001). HNE is capable of exhibiting a wide range of biological effects from alteration in gene expression and cell signaling, to cell proliferation and apoptosis (Parola et al., 1999; Poli and Schaur, 2000). The presence of high levels of HNE and HNE protein adducts has been implicated in a number of human diseases caused by oxidative stress, including Alzheimer's disease,

Parkinson's disease, arteriosclerosis, and hepatic ischemia reperfusion injury (Sayre et al., 1997); (Napoli et al., 1997; Yamagami et al., 2000; Yoritaka et al., 1996), thus making HNE one of the most widely studied lipid peroxidation products (Parola et al., 1999; Poli and Schaur, 2000).

HNE can be metabolized in various ways. It can be oxidized to 4-hydroxynon-2-enoic acid (4HNA) by mitochondrial aldehyde dehydrogenase (ALDH2) (Mitchell and Petersen, 1987). It can be reduced via the action of numerous enzymes, including members of the aldo-keto reductase family and/or alcohol dehydrogenase, yielding the inactive 1,4-dihydroxy-2-nonene (DHN) (Burczynski et al., 2001; O'Connor et al., 1999; Srivastava et al., 1999; Vander Jagt et al., 1995) . GSH can react with HNE via Michael addition to form a glutathione-HNE conjugate. This reaction is catalyzed by glutathione-S-transferase (Alin et al., 1985). Enzyme catalyzed reduction of the C=C of HNE can be brought about by alkenal/one oxidoreductase (AO) to yield 4-hydroxynonanal (4HAA), which rearranges to a lactol form that does not contain a free aldehyde (Dick et al., 2001). These reactions are illustrated in Fig. 4.1. In rat hepatocytess GSH conjugation was the major pathway, representing 50-60% of HNE elimination whereas oxidation/reduction of HNE was found to account for only 10% of the metabolism, while the remaining products (30%-40%) were not identified (Hartley et al., 1995).

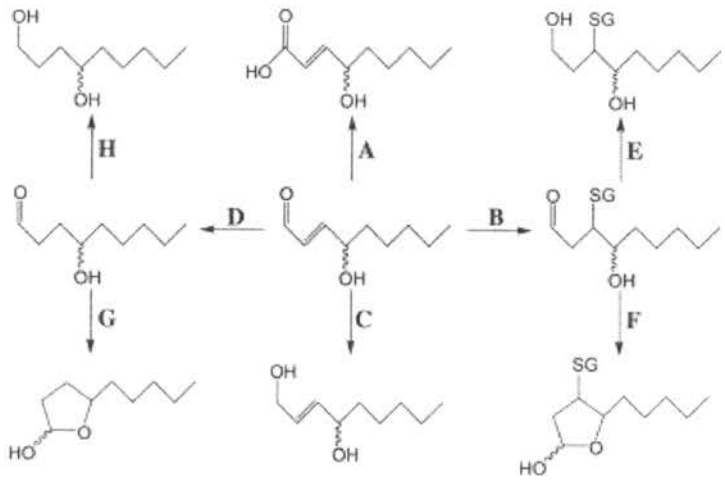


Fig 4.1. Major pathways for 4HNE biotransformation. (A) ALDH2-catalyzed oxidation to 4HNA. (B) Spontaneous or GST-mediated GSH conjugation yielding GS–4HNE. (C) Enzyme-catalyzed reduction to DHN. (D) AO-mediated reduction of C=C yielding 4HAA. (E) Aldose reductase-catalyzed reduction to GS-DHN. (F) Spontaneous rearrangement to a cyclic hemiacetal adduct. (G) Spontaneous rearrangement to a lactol. (H) Possible reduction of 4HAA. Taken from (Petersen and Doorn, 2004)

HNE is proposed to be further metabolized to 2,3-epoxy-4-hydroxynonenal, which in turn can react with DNA to generate different etheno adducts (Chen and Chung, 1994; Sodum and Chung, 1989). However, epoxidation is not known to occur in the *in vivo* metabolism of HNE (Alary et al., 1995; Alary et al., 1998). Also, the etheno adducts that are detected *in vivo* are not HNE-specific and can be derived from other compounds (Bolt, 1988; Bolt, 1994). Thus, the propano deoxyguanosine adducts may constitute the most significant DNA adducts formed from HNE.

To date, the mutagenic potential of the individual propano HNE-dG adducts has not been established. The first step in accomplishing such a goal was to obtain site and stereospecifically adducted DNAs. Recently, oligodeoxynucleotides containing the four stereoisomers of HNE, *(6R, 8S, 11R), (6S, 8R, 11S), (6R, 8S, 11S),* and *(6S, 8R, 11R),* adducted at 1,N^2-deoxyguanosine, have been synthesized as shown in Fig. 4.2 (Wang et al., 2003). In this study, I analyzed the ability of these HNE-dG stereoisomers to induce mutations in mammalian cells.

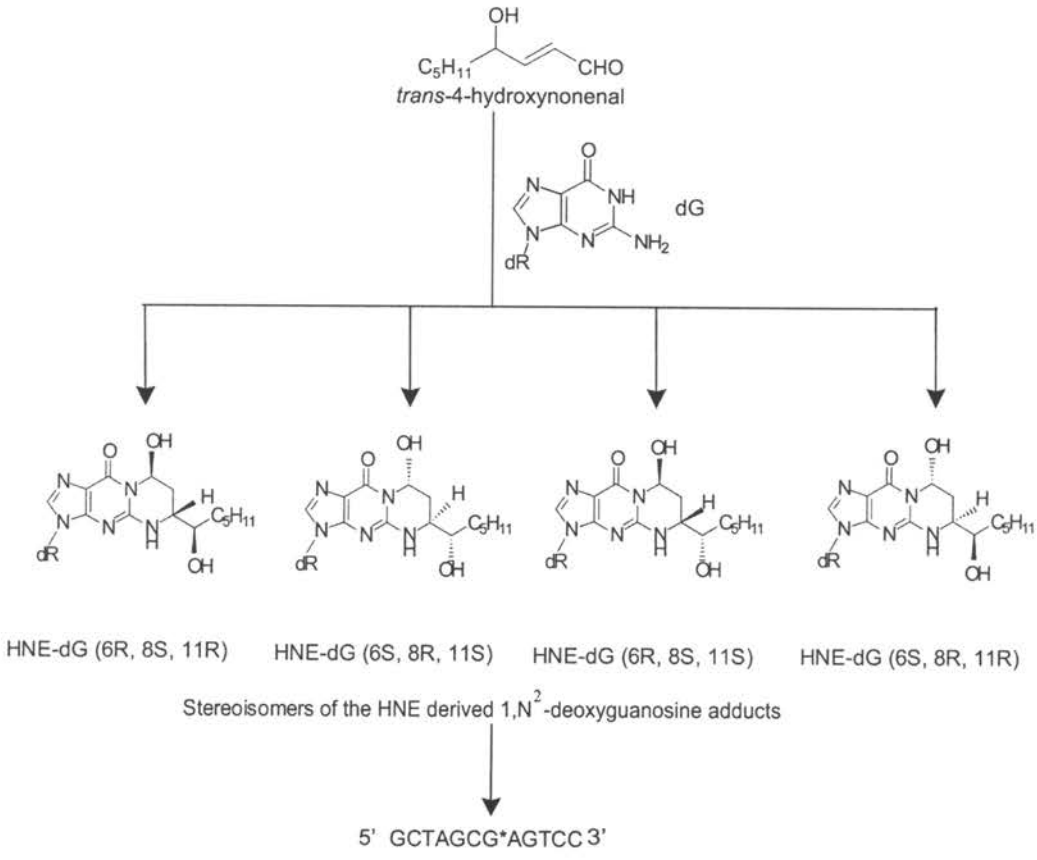


Fig. 4.2. Formation of the four stereoisomeric HNE-dG adducts from the reaction of deoxyguanosine with HNE and introduction of these adducts into specific 12-mer oligodeoxynucleotides.

MATERIALS AND METHODS

Materials. ss pMS2 DNA was a generous gift from Dr. M. Moriya (State University of New York, Stony Brook, NY). T4 DNA ligase, T4 polynucleotide kinase, and *Eco*RV were purchased from New England BioLabs (Beverly, MA); and S1 nuclease was obtained from Invitrogen (Carlsbad, CA). COS-7 cells were obtained from American Type Culture Collection (Rockville, MD). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, L-glutamine, and antibiotic-antimycotic, Opti-MEM (reduced serum medium) and lipofectin reagent for transfection were obtained from Invitrogen. Trypsin-EDTA was purchased from Cellgro Mediatech (Herndon, VA) and phosphate-buffered saline was obtained from Sigma (St. Louis, MO). Bio-Spin columns were purchased from Bio-Rad (Hercules, CA), while Centricon 100 concentrators were acquired from Amicon (Beverly, MA). [γ^{32}P] Ribo-ATP was purchased from Perkin Elmer Life Sciences (Boston, MA). *E. coli* DH10B cells, used for transformation of progeny plasmid DNA from COS-7 cells, were purchased from Invitrogen.

Synthesis of HNE modified oligodeoxynucleotides. The following work was carried out by Wang and Dr. Rizzo at Vanderbilt University, Tennessee. The HNE-derived 1,N^2-deoxyguanosine adducts, *(6R, 8S, 11R), (6S, 8R, 11S), (6R, 8S, 11S)* and *(6S, 8R, 11R)*, were synthesized by a post-synthetic modification of the oligodeoxynucleotide 5'-GCTAGCXAGTCC-3', where X is 2-fluoro-O^6- (2-trimethylsilylethyl)-2'-deoxyinosine. Reaction of this oligodeoxynucleotide with the appropriate stereochemically defined aminotriol (Wang and Rizzo, 2001), followed by treatment with sodium periodate, gave the desired site-specifically modified oligodeoxynucleotides, 5'-GCTAGC**G***AGTCC-3' where **G***, is one of the four HNE-dG adducts (Fig. 4.2). This strategy has also been

successfully used for the synthesis of related 1,N^2-deoxyguanosine adducted oligodeoxynucelotides (Kozekov et al., 2003; Nechev et al., 2000; Nechev et al., 2001). Adducted oligodeoxynucleotides were purified by reverse phase HPLC both before and after treatment with sodium periodate. HPLC analysis of an enzyme digest and MALDI-MS (mass spectrometry) confirmed that the adduct had been incorporated. Further details on the synthesis and analyses of these oligodeoxynucleotides were published elsewhere (Wang et al., 2003). The control oligodeoxynucleotide consisted of the same sequence, except that an unmodified dG replaced the adducted guanosine (**G***). The unmodified sequence was purchased from Midland Certified Reagent Co. (Midland, TX).

Construction of circular single-stranded pMS2 DNAs containing the 1,N^2-deoxyguanosine adducts of HNE. The methods for introducing DNAs containing defined lesions into the ss pMS2 vector (Moriya, 1993) were performed as described by Kanuri et al., (2002). In brief, ss pMS2 (59 pmol, 100 μg) was annealed to a 58-mer scaffold (295 pmol, 500 μg), and the annealed product digested with *Eco*RV to release the hairpin loop structure of ss pMS2. This procedure results in the creation of a precise gap in the DNA. The scaffolding DNA was designed such that 22 nucleotides on the 5′ end and 24 nucleotides on the 3′ end were complementary to the two termini of the digested ss pMS2. The 58-mer also had a central sequence that was complementary to the 12-mer oligodeoxynucleotide, bearing one of the HNE-dG adducts or the unmodified guanosine. To ensure that scaffolding DNA was not used as a substrate for *in vivo* replication, all thymines were substituted by uracil. The HNE-modified 12-mers and the control 12-mer were phosphorylated at the 5′ end using T4 DNA kinase (5 units/pmol) and then ligated (325 units of T4 DNA ligase/pmol) into the partially duplex pMS2

vector. The ligated DNAs were then purified in Centricon 100 concentrators (to remove unligated 12-mers) and recovered by ethanol precipitation. The products obtained were treated with uracil DNA glycosylase for 1 h to severely damage the scaffold DNA and prevent it from being used as a primer for (-)- strand replication. For verification of ligation, aliquots of the ligation products were subjected to electrophoresis through a 1.4% agarose gel in 1X TAE buffer (40mM Tris acetate, 1mM EDTA), stained with ethidium bromide (0.5 μg/mL), and compared with the standard circular and linearized ss pMS2 DNA.

Site-specific mutagenesis in COS-7 cells. The overall strategy for replication and mutagenic analyses was as previously described in Chapter 2.

RESULTS

In order to determine if the replication of DNAs containing 1,N^2-deoxyguanosine HNE adducts with known stereochemistry results in mutations, 12-mer DNAs carrying these adducts or control deoxyguanosine were engineered into a ss DNA shuttle vector, replicated in COS-7 mammalian cells and the resultant DNAs analyzed for mutations by differential hybridization strategies. The HNE adducted DNAs used in our mutagenesis *assay are very stable both at the nucleoside level and within single-stranded DNAs, with* no indication of depurination at the HNE sites. Therefore any mutations that are observed are due to the presence of these HNE-dG adducts. Typical results obtained, through differential hybridization and autoradiography of the individually picked colonies, are as shown in Fig. 4.3.

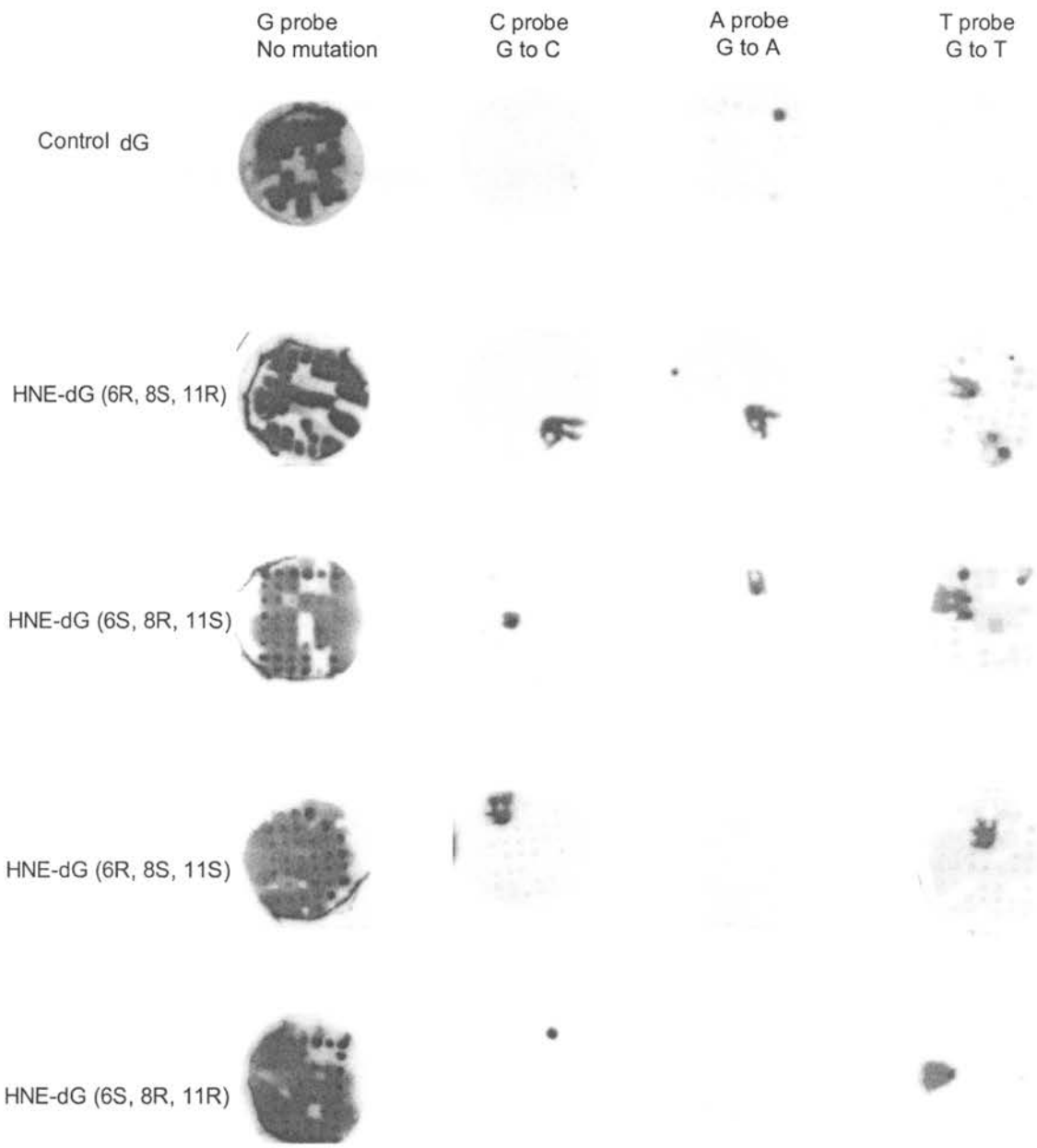


Fig. 4.3. Autoradiographs displaying the result of non-mutagenic and mutagenic replication past the HNE-dG adducts in COS-7 cells. ss pMS2 vector containing the specific HNE adduct or control dG was used to transfect COS-7 cells and allowed to replicate for 48 h. The progeny plasmids were then recovered from the COS-7 cells and

used to transform *E. coli* DHB10 cells. Individual transformants were picked and grown in 96 well plates containing LB with 100 μg/mL ampicillin. The resultant colonies were transferred in four replicates onto Whatman 541 filter papers, one for each of the 4 probes G, C, A and T; and were differentially hybridized.

The colonies represent progeny plasmid DNAs that were analyzed with each of the four probes (5'-GATGCTAGC**N**AGTCCATC-3' where **N** refers to G, C, A or T) to determine the nature of point mutations or deletions that were formed during replication in COS-7 cells. Approximately 500 colonies, a number that is sufficient to obtain statistically significant data, were picked and analyzed for each HNE-dG adduct and control. However, for both the control and HNE-dG adducts some of the colonies failed to undergo hybridization with any of the four probes due to the loss of the probe binding sequence, which occurs during the replication cycles of the progeny plasmid DNA. Therefore, only the colonies that hybridized with any of the four probes were included for calculation of the mutation yield. Three independent ligations and transfections into COS-7 cells of the control and modified DNAs were carried out and the combined results are shown in Table 4.1.

ss pMS2 adduct	Number of mutations detected				Total Mutation Yield (%)
	None	G → C	G → A	G → T	
Control dG	337	0	1	0	0.3
HNE-dG *(6R, 8S, 11R)*	326	2	5	10	5
HNE-dG *(6S, 8R, 11S)*	335	1	2	11	4
HNE-dG *(6R, 8S, 11S)*	379	1	0	1	0.5
HNE-dG *(6S, 8R, 11R)*	350	1	0	3	1.1

Table 4.1. Mutation yield of the HNE derived 1,N^2-deoxyguanosine adducts in COS-7 cells

The method of individually picked colonies (Fig. 4.3 and Table 4.1) indicates that (*6R, 8S, 11R*)-HNE and (*6S, 8R, 11S*)-HNE adducts were significantly more mutagenic than the (*6R, 8S, 11S*)-HNE and (*6S, 8R, 11R*)-HNE adducts. The predominant mutations for the (*6R, 8S, 11R*)-HNE and (*6S, 8R, 11S*)-HNE adducts were the G to T transversions, with no evidence for adduct-induced deletions. Analyses of the replication products of DNAs carrying the control dG, showed a single G to A mutation from 338 hybridized control colonies (Table 4.1). No prior studies using control vectors have revealed point mutations (Kanuri et al., 2002a); this isolated event could be due to low levels of background mutagenesis inherent within this *in vivo* system. In the case of the individually picked colonies, those indicative of mutation were selected for plasmid extraction and DNA sequence analyses. The sequencing data confirmed the nature of the mutations revealed through autoradiography.

The data in Table 4.1 was subjected to Fisher's exact test, where both the individual mutations and the total mutation yield for each adduct was compared to the control and tested for homogeneity, i.e. if the mutant populations of the control and HNE-dG adducts were statistically the same or different. The *p* values so obtained, indicated that the mutation yields of the (*6R, 8S, 11R*)-HNE and (*6S, 8R, 11S*)-HNE adducts were statistically different from control, whereas those of the (*6R, 8S, 11S*)-HNE and (*6S, 8R, 11R*)-HNE adducts were not. Further, the G to T mutations for the (*6R, 8S, 11R*)-HNE and (*6S, 8R, 11S*)-HNE adducts were statistically different from control.

DISCUSSION

HNE, the principal α, β-unsaturated aldehydic end product of lipid peroxidation, can elicit multiple interactions with cellular components, including DNA. The deoxyguanosine adducts of HNE occur as inherent lesions in rodents and their frequency is increased upon treatment with HNE or induction of lipid peroxidation (Chung et al., 2000; Wacker et al., 2001). The system that I employed, analyzed the ability of the replication complexes within mammalian cells to bypass site-specific and stereochemically-defined HNE-dG adducts. My results show that these HNE-dG adducts are differentially mutagenic in that the (*6R, 8S, 11R*) and (*6S, 8R, 11S*) HNE-dG adducts induced at least 4-fold greater point mutations than the (*6R, 8S, 11S*) and (*6S, 8R, 11R*) stereoisomers. Melting studies using adducted oligodeoxynucelotides annealed to complementary 12-mers showed the same differential pattern, in that the (*6R, 8S, 11R*) and (*6S, 8R, 11S*) HNE-dG adducts were highly destabilizing and caused a significant decrease in Tm values, as compared to the control and (*6R, 8S, 11S*) and (*6S, 8R, 11R*) stereoisomers (Wang et al., 2003).

The C11-stereochemistry of the HNE-dG adducts is derived from HNE, which is presumably produced in racemic form from lipid peroxidation. Thus, the (*6R, 8S, 11R*)-HNE and (*6S, 8R, 11R*)-HNE adducts are derived from the *R*-enantiomer (4*R*-HNE) and the (*6S, 8R, 11S*)-HNE and (*6R, 8S, 11S*)-HNE adducts are derived from the *S*-enantiomer (4*S*-HNE). My results suggest that the 4*R*-HNE and the 4*S*-HNE would give roughly equal frequencies of point mutation, if each diastereomeric adduct were formed equally. The reaction of racemic HNE with calf thymus DNA and analysis of the adducts by ^{32}P-postlabeling has been reported (Yi et al., 1997). Interestingly, only two of the four

possible stereoisomers were observed, with (*6S, 8R, 11S*)-HNE and (*6R, 8S, 11R*)-HNE being the major product and minor product respectively. Although these two stereoisomers were the most mutagenic in my assays, it should be pointed out that all four stereoisomers have been observed from ^{32}P-analysis of DNA samples from animal tissue (Chung et al., 2000).

Of the mutations detected, there were strong preferences for two of the diastereomers [*(6R, 8S, 11R)*-HNE and *(6S, 8R, 11S)*-HNE adducts] to induce G to T transversions (Table 4.1). These observations are consistent with other studies that suggest formation of HNE-dG adducts at the third base of codon 249 (-AGG*-) in the *p53* gene leads to a high frequency of G to T transversions (Hu et al., 2002; Hussain et al., 2000).Therefore, spatial disposition of the stereochemical groups appears to govern mutagenesis and influences the polymerase's ability to bypass the HNE-adduct in a mutagenic or non-mutagenic fashion.

Other factors that influence the mutagenic potential of each adduct could be the *anti* or *syn* orientation of the base around the glycosidic bond, or ring-opened or ring-closed forms. The HNE-dG adducts are hypothesized to undergo ring-opening in duplex DNA, thus displacing the aldehydic moiety in the minor groove and facilitating conventional Watson-Crick base pair formation with an incoming deoxycytosine (Fig. 4.4). This correct base-pairing could account for the high number of non-mutagenic substitutions observed with the four HNE stereoisomers.

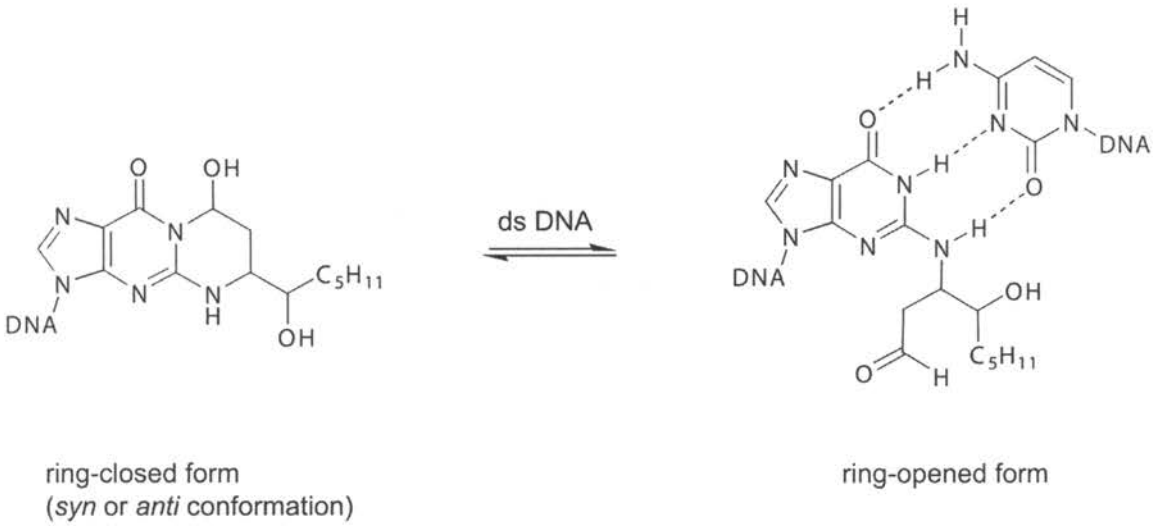


Fig. 4.4. Ring-closed and ring-opened forms of the HNE-dG adduct

The hypothesis of the ring-open/closed form of the adduct affecting mutagenicity is based on recent studies of the structurally related acrolein-deoxyguanosine adduct, γ-HOPdG. In double-stranded (ds) DNA this adduct exists primarily in the ring-opened form due to incorporation of a correct nucleotide C opposite it (de los Santos et al., 2001). Therefore ring-opening of the adduct is associated with it being non-mutagenic. The *(6R, 8S, 11R)*-HNE and *(6S, 8R, 11S)*-HNE adducts would be predicted to be in the ring-closed form, based on their higher mutation yield. However *in vitro* DNA-peptide (when the reaction is carried out in the presence of $NaCNBH_3$) and DNA-DNA cross-linking studies indicate otherwise. These studies demonstrated that the 12-mer duplex oligodeoxynucleotide containing the *(6S, 8R, 11S)*-HNE adduct forms significant levels of both DNA-peptide cross-links (Kurtz and Lloyd, 2003) and DNA-DNA cross-links (Wang et al., 2003), which is possible only if the *(6S, 8R, 11S)*-HNE adduct undergoes ring-opening. These differences in mutagenicity vs. cross-linking can be reconciled due to time differences between the conditions of *in vitro* cross-linking and *in vivo* replicaton; and the predominant ring-closed structure of the HNE-dG adduct in ss DNA. The differential (ring-opened/ring-closed) structures of these exocyclic adducts in ss vs. ds DNA is evident from site-specific mutagenicity studies of the related γ-HOPdG and α-hydroxy-1,N^2-propano-2′-deoxyguanosine (α-HOPdG) adducts, the latter adduct is unable to undergo ring-opening. In double stranded vectors, there was a 30-fold difference in the mutagenicity of γ-HOPdG vs. α-HOPdG (Yang et al., 2002), whereas in the single-stranded vectors, there was no significant difference in the mutagenicity of γ-HOPdG vs. α-HOPdG (Kanuri et al., 2002a; Sanchez et al., 2003).

HNE is derived from ω-6 polyunsaturated fatty acids such as arachidonic acid and linoleic acid (Esterbauer et al., 1991) that are known to promote metastasis and increase the risk of certain human cancers like colon and breast cancer (Bartsch et al., 1999). My studies demonstrate that the mutagenic capability of HNE is due to the formation of the *(6R, 8S, 11R)*-HNE and *(6S, 8R, 11S)*-HNE adducts, thus suggesting that formation of these adducts is a potential pathway leading to tumorigenesis. Studies show that the repair of the HNE-dG adducts involves the NER pathway, in that the *E. coli* UvrABC nuclease complex is capable of incision and removal of DNA containing HNE-dG adducts (Hu et al., 2002). Therefore, the actual levels of HNE-dG adducts detected in *in vivo* studies represent a balance between the generation and repair of adducts, which may explain the increased levels of HNE modified proteins, but not HNE-dG adducts in the brains of Alzhemier's patients (Gotz et al., 2002). In conclusion, the stereospecific *(6R, 8S, 11R)*-HNE and *(6S, 8R, 11S)*-HNE adducts account for the mutagenicity of HNE and may serve as specific biomarkers for tumorigenesis.

ACKNOWLEDGEMENTS

I acknowledge Dr. Masaki Moriya, Department of Pharmacological Sciences, University of New York at Stony Brook, for the generous gift of the ss pMS2 vector, Dr. Judah Rosenblatt, University of Texas Medical Branch, for the statistical analysis and Dr. Manorama Kanuri for helpful discussions.

CHAPTER 5

MAMMALIAN CELL MUTAGENESIS OF THE DNA ADDUCTS OF VINYL CHLORIDE AND CROTONALDEHYDE

ABSTRACT

Vinyl chloride and crotonaldehyde are known mutagens and carcinogens, through their reaction with DNA to form specific deoxyguanosine adducts. To investigate the mutagenic potential of a subset of the possible deoxyguanosine lesions, site-specific adducts of vinyl chloride and crotonaldehyde were synthesized, inserted into a shuttle vector and replicated in mammalian cells. Mutation yields of the DNA adducts of vinyl chloride and crotonaldehyde were found to be 2% and 5-6% respectively, thus suggesting that these adducts could contribute to the overall genotoxicity and carcinogenicity associated with exposure to these chemicals.

INTRODUCTION

Bifunctional aldehydes play a prominent role in the field of DNA mutagenesis and carcinogenesis due to their ubiquitous nature, and their capacity to react with DNA to form adducts that may subsequently block replication or induce mutations. Some of the common DNA damaging, bifunctional aldehydes include acrolein, crotonaldehyde, 4-hydroxynonenal, malondialdehyde and chloroacetaldehyde (Chung et al., 1999; Marnett, 1999; Singer et al., 2002).

DNA adduct-induced mutagenesis is dependent on various factors that include, but are not limited to the chemical structure of the adduct, the sequence context of the lesion, and the stereochemistry, and conformation of the adduct that is present in the replication fork complex. In this study, the cellular mutagenicity of two structurally related DNA adducts was compared, with the goal of gaining insight into how structural differences can be correlated with differences in mutagenic potential. The adducts under investigation are the β-HOEdG adduct derived from vinyl chloride and the *R* and *S* α-Me-γ-HOPdG adducts derived from crotonaldehyde (Fig. 5.1).

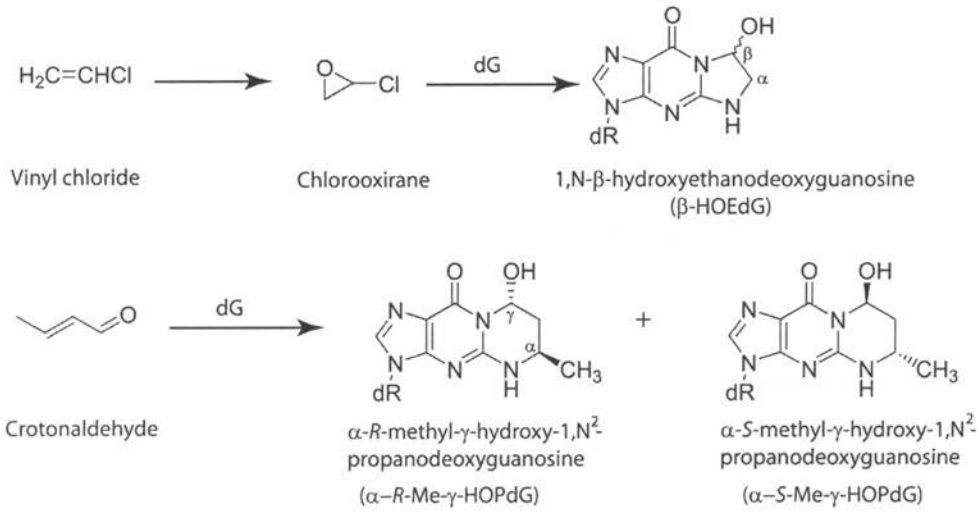


Figure 5.1. Formation of the β-HOEdG and α-Me-γ-HOPdG adducts from vinyl chloride and crotonaldehyde, respectively.

Vinyl chloride (CH_2=CHCl) is an industrial chemical that is both a human and animal carcinogen, known for causing angiosarcomas of the liver (McLaughlin and Lipworth, 1999; Purchase et al., 1987). Its metabolic activation proceeds sequentially to chlorooxirane and chloroacetaldehyde, both of which can react with DNA to form various etheno and ethano DNA adducts. While the etheno adducts are known to be excised by specific glycosylases (Gros et al., 2003), little is known about the mutagenic potential and repair of the stable β-HOEdG adduct of vinyl chloride.

Crotonaldehyde (CH_3CH=CHCHO) is an important industrial chemical, an environmental pollutant and a byproduct of lipid peroxidation (Marnett, 1994; Pan and Chung, 2002). It is both genotoxic and mutagenic in a human lymphoblast cell line (Czerny et al., 1998) and induces liver tumors in rodents (Chung et al., 1986). Its primary reactivity with DNA bases is through guanine in which two major regiosomeric adducts are formed and denoted as the α*R*- and α*S*-Me-γ-HOPdG adducts, where the hydroxyl group is proximal to the N1 position of guanine (Eder and Hoffman, 1992). These adducts have been detected in human and rodent tissues with or without exposure to exogenous crotonaldehyde (Budiawan and Eder, 2000; Chung et al., 1999; Yang et al., 1999).

Increasing evidence of the mutagenicity and genotoxicity of vinyl chloride and crotonaldehyde makes it important to evaluate the mutagenic potential of the adducts derived from them, namely the β-HOEdG and the α*R*- and α*S*-Me-γ-HOPdG adducts, which to date have not been studied. Structurally related adducts, PdG and γ-HOPdG, are mutagenic (Benamira et al., 1992; Kanuri et al., 2002a; Moriya et al., 1994), suggesting that these adducts may also induce mutations. The syntheses of adducted

oligodeoxynucleotides containing β-HOEdG and the α-Me-γ-HOPdG diastereomers (Nechev et al., 2000; Nechev et al., 2001) have allowed us to evaluate their mutagenic potential in mammalian cells, as described herein.

MATERIALS AND METHODS

The synthesis of nucleosides and oligodeoxynucleotides containing the 1,N^2 dG adducts of vinyl chloride and crotonaldehyde are described elsewhere (Nechev et al., 2000; Nechev et al., 2001). The β-HOEdG and α-Me-γ-HOPdG adducts were prepared in the following sequence context 5′-GCTAGC**G***AGTCC-3′ where **G*** represents the adducted guanine. The mutational analysis of the α-Me-γ-HOPdG adducts was performed by Dr. Manorama Kanuri. An undamaged 12-mer oligodeoxynucleotide containing a dG in place of the adducted guanine was purchased from Midland Certified Reagent Co. (Midland, TX). These oligodeoxynucleotides were ligated into the ss shuttle vector, pMS2, as described previously (Kanuri et al., 2002) and subsequently transfected into COS-7 mammalian cells. Transfection efficiencies were estimated to be around 10-15% using a GFP expression vector. Following replication, these cells were harvested and the resulting progeny plasmid DNA was isolated as a Hirt supernatant (Hirt, 1967). In order to evaluate the mutagenic or non-mutagenic events that are the result of replication past the adducts, the progeny plasmid DNAs were then used to transform DH10B *E. coli* cells, which were subsequently selected on LB-ampicillin plates (100 μg/ml). The resulting colonies were individually picked and grown overnight in 96 well formats. The cultures were transferred onto Whatman 541 filter papers in four replicates, incubated overnight and subjected to differential DNA hybridization using the four probes, 5′-ATGCTAGC**N**AGTCCATC-3′, where **N** refers to G, C, A or T (Kanuri et al., 2002). For further verification, the colonies indicative of mutations by autoradiography were then selected and processed for DNA sequence analysis.

RESULTS AND DISCUSSION

The site-specifically adducted oligodeoxynucleotides used in these studies were prepared by published procedures and were exhaustively purified and the purity carefully documented in order to assure that mutagenesis results would reliably reflect the misincorporation potential of the lesions being investigated. The syntheses utilized a post-oligomerization procedure in which condensations of a 12-mer containing O^6-trimethylsilylethyl-protected 2-fluoro-2′-deoxyinosine with the appropriate aminodiols were followed by sodium periodate cleavage of the resulting N^2-(dihydroxyalkyl) chains to aldehydes which spontaneously cyclized to the pyrimidopurinones. The oligodeoxynucleotides containing α*R*- and α*S*-Me-γ-HOPdG were prepared from the racemic aminodiol and the resulting diastereomers were chromatographically separated at the final stage. HPLC purification was carried out at each stage in the synthesis, i.e., the oligodeoxynucleotides containing the O^6-trimethylsilylethyl-protected 2-fluoro-2′-deoxyinosine, the N^2-(dihydroxyalkyl)-2′-deoxyguanosines, and the β-HOEdG and α*R*- and α*S*-Me-γ-HOPdG final products. MALDI-MS confirmed the products had the expected molecular weights. Enzymatic digestion of the final oligodeoxynucleotides gave the appropriate ratios of the five constituent nucleosides and showed the oligodeoxynucleotides to be free of aberrant nucleosides. CZE was used to obtain a quantitative determination of purity. By this method, the purities of the oligodeoxynucleotides containing β-HOEdG and α*R*- and α*S*-Me-γ-HOPdG were shown to be 98.5, 99.5, and 99.9%. The impurities in the first two samples were most likely the truncated oligodeoxynucleotides, which would subsequently be discriminated against during ligation into the shuttle vector.

The adducted vectors were subsequently replicated in mammalian cells and the resultant progeny DNAs were evaluated for mutations using differential hybridization. The results obtained from autoradiography are shown in Fig. 5.2.

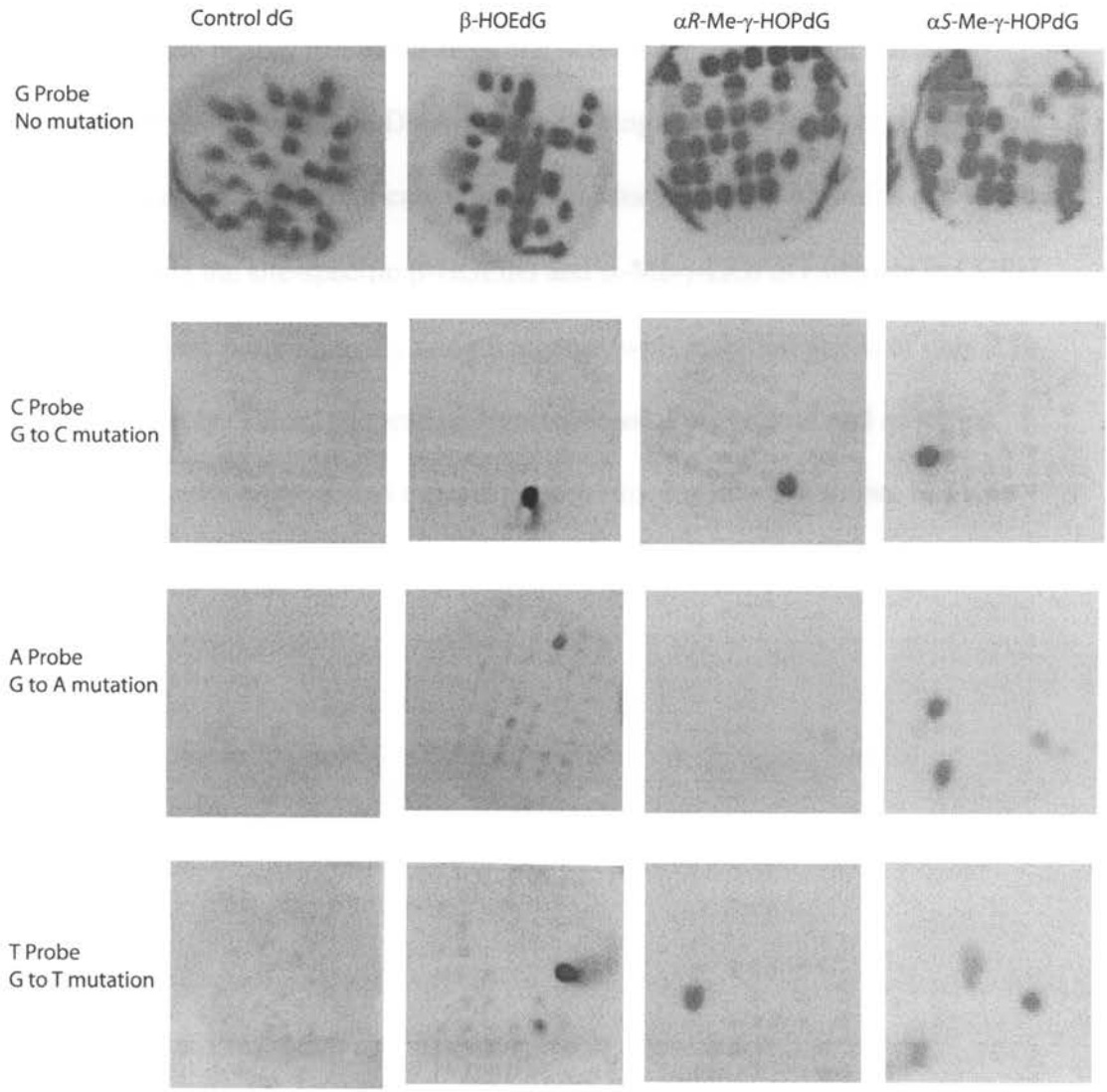


Figure 5.2. Autoradiographs indicating single base substitution mutations due to β-HOEdG and α-Me-γ-HOPdG adducts.

The dark colonies represent progeny DNA that annealed to one of four oligodeoxynucleotide probes that differ from one another by only a single centrally-located nucleotide within the probe DNA, thus indicating the occurrence of a mutagenic or non-mutagenic event during replication past the adduct. Progeny plasmids that resulted from replication past the site-specific β-HOEdG and α-Me-γ-HOPdG adducts in COS-7 mammalian cells were predominantly non-mutagenic, with mutation yields of only 2 % and 5-6 % respectively. Three independent transfections of the control and modified DNAs into COS-7 cells were carried out and the combined results are shown in Table 5.1.

ss pMS2 vector containing	Number of mutations detected				Mutation yield (%)
	None	G → C	G → A	G → T	
Undamaged dG	662	0	0	0	0
β-HOEdG	653	2	1	11	2
α*R*-Me-γ-HOPdG	343	4	2	11	4.7
α*S*-Me-γ-HOPdG	302	3	4	13	6.2

Table 5.1. Mutation spectrum during replication past β-HOEdG, α*R*-Me-γ-HOPdG, and α*S*-Me-γ-HOPdG adducts in COS-7 cells

G to T transversions were the predominant mutation for the three adducts, followed by a much lower number of G to C and G to A mutations. The β-HOEdG adduct appeared to be less mutagenic than the α-Me-γ-HOPdG adducts. The *S* adduct of crotonaldehyde was slightly more mutagenic than the *R*, although the pattern of mutations between the two diastereomers was very similar.

The spectrum of mutations generated from the replication of the α-Me-γ-HOPdG adducts in this study compared favorably with data previously acquired using a shuttle vector that had been randomly modified with crotonaldehyde and replicated in human cells, in which the highest frequency of mutations were the G to T transversions (Kawanishi et al., 1998). Additionally, the spectrum but not the frequency of mutations seen in our analyses with the β-HOEdG adduct is in agreement with Langouet et al., (1998), who showed that the mutation rate for this adduct was no more than 0.1% for G to T or G to A or G to C in SOS-induced *E. coli* cells.

The mutation frequencies for both these exocyclic adducts are generally much higher in mammalian cells than data obtained in *E. coli* (Langouet et al., 1998); Kanuri et al., unpublished data). These data are consistent with other site-specific mutagenesis studies that had investigated related unmethylated propano adducts, using the same sequence context and vector. Those analyses revealed higher mutation yields in mammalian cells than in *uvr*A$^-$ *rec*A$^-$ *E. coli* cells that were not SOS induced (Fernandes et al., 2003; Kanuri et al., 2002a). These data suggest the presence of a more accurate bypass system of propano deoxyguanosine and related adducts in *E. coli*. However, these data have been acquired using single-stranded shuttle vectors in which there should be no opportunity to repair the lesion prior to replication to form duplex DNA. However, if

these lesions were formed in duplex DNA, repair systems would have the opportunity to remove the lesions prior to mutagenic bypass. Although no data have been collected for the repair of the β-HOEdG and α-Me-γ-HOPdG adducts, some of the propano adducts are known to be repaired through NER (Feng et al., 2003; Yang et al., 2001). These data suggest that repair of the structurally related β-HOEdG and α-Me-γ-HOPdG adducts may also occur by the same process.

Previous *in vitro* DNA replication studies using Kf exo$^-$ have shown that synthesis was blocked beyond the site of the DNAs containing the β-HOEdG adduct and with misincorporation of dATP and dGTP opposite the site of the adduct (Langouet et al., 1997). The *R* and *S* adducts of crotonaldehyde severely blocked synthesis by both Kf exo$^-$ and pol ε (Kanuri et al., unpublished data). Additionally studies by Washington et al. showed that pol ι in conjunction with pol κ, and Rev 1 in combination with pol ζ could efficiently bypass the structurally related γ-HOPdG adduct (Washington et al., 2004b; Washington et al., 2004c); whereas pol η could bypass the γ-HOPdG lesion to a lesser extent (Minko et al., 2003). It is plausible that these translesion polymerases are also capable of bypassing the β-HOEdG and α-Me-γ-HOPdG adducts.

Mutagenesis by the β-HOEdG adduct of vinyl chloride is reduced in comparison with that induced by the related 1,N^2etheno guanine adduct, which differs structurally through the absence of a hydroxyl group (Akasaka and Guengerich, 1999). This may be explained by the differential structure of the adduct at the replication fork. While the etheno adduct is permanently in a ring-closed form, the β-HOEdG exists in an equilibrium between its ring-closed and ring-opened forms (Langouet et al., 1997). A NMR study of its propano homolog, γ-HOPdG indicated that the ring-opened form of this

adduct is favored in duplex DNA by correct base-pairing (de los Santos et al., 2001). This finding has been proposed to account for its lower mutagenicity relative to its structurally fixed counterpart, PdG, which lacks the hydroxyl group needed for ring opening. This capacity to form stable ring-opened lesions may help to explain why the β-HOEdG adduct is less mutagenic than the permanently ring-closed 1,N^2etheno guanine adduct. The same explanation could account for the low mutagenicity of the *R* and *S* adducts of crotonaldehyde in which there already exists a precedence for the ring-opened form of the crotonaldehyde adduct in duplex DNA by its virtue of its ability to form DNA-DNA and DNA-peptide cross-links (Kurtz and Lloyd, 2003). Comparison of the relative reactivities of the *R* and *S* crotonaldehyde adducts to conjugation with peptides gave identical results, suggesting that both undergo ring-opening equally well.

γ-HOPdG-induced mutagenesis appears to be higher than that caused by the β-HOEdG and α-Me-γ-HOPdG adducts in mammalian cells using the same vector and sequence context (Kanuri et al., 2002). This observation indicates a few possibilities. First, these adducts may be blocking replication, thus preventing a higher level of misincorporation events opposite the adducted sites as seen with γ-HOPdG. Second, the 2-carbon hydroxylated β-HOEdG may be structurally less distorting than the 3-carbon hydroxylated γ-HOPdG, and in the case of the α-Me-γ-HOPdG adducts, the additional methyl group may facilitate a higher number of error-free bypass events than that seen with γ-HOPdG.

Therefore the β-HOEdG and α-Me-γ-HOPdG adducts are mutagenic and may contribute to the carcinogenesis associated with their respective parent chemicals.

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CHAPTER 6

SUMMARY AND CONCLUSIONS.

The global demand for BD has dramatically risen over the past two decades and continues to do so, in order to supply the consumers' need for rubber-based goods (Fajen et al., 1990). This in turn poses a risk to occupational workers at butadiene plants. The fact that butadiene is present in automobile exhaust, cigarette smoke and gasoline vapor also subjects the general population to its hazardous effects (Brunnemann et al., 1990; Pelz et al., 1990). Epidemiological studies indicate a positive association between exposure to butadiene and increased incidence of leukemia, but do not prove carcinogenicity in humans (Delzell et al., 1996; Graff et al., 2005; Macaluso et al., 1996; Santos-Burgoa et al., 1992). The differences in the metabolism of BD in humans and rodents have made it difficult to extrapolate the risk to humans, although BD is an established rodent carcinogen. Thus a critical gap in the knowledge base still exists, that centers on how butadiene metabolites cause DNA damage that ultimately results in human tumorigenesis. This gap has led butadiene to be categorized as a probable and not a definitive human carcinogen. Therefore, this paucity of information warrants a closer examination of the molecular mechanisms underlying butadiene-mediated carcinogenesis. My approach has been to analyze DNA adducts formed from the metabolites of BD, and more specifically, I have examined the role of the BD N3-dU adducts in BD-mediated mutagenesis.

The study of these DNA adducts has been made possible through the synthesis of the adducted oligodeoxynucleotides. These adducts were then examined for their mutagenic potential in mammalian cells using a single-stranded shuttle vector. The focal point of my research has been the finding of 97% mutagenicity with replication past these adducts in mammalian cells. The only other BD-derived DNA adduct close to this

mutagenic rate is the N1-deoxyinosine adducts (Kanuri et al., 2002b). Although the BD N3-dU adducts are derived from uracil, they exhibit a different mutational spectrum than that of uracil. Along with the expected C to T mutations, C to A and C to G mutations were also induced in mammalian cells. This is in agreement with the fact that GC to AT mutations are known to be associated with EB (Vanduuren et al., 1963), the metabolite from which the BD N3-dU adducts are derived. The BD N3-dU adducts represent a mixture of the R and S isomers. It would be interesting to see if the individual isomers reflected a differential mutation pattern. This is exhibited with the N1 deoxyinosine adducts where there was a difference not only in the mutagenicity of the *R* and *S* isomers, but also in the pattern of mutations induced by them (Kanuri et al., 2002b).

Having established that the BD N3-dU adducts can be bypassed in cells, the next step was to identify which polymerase was responsible for this bypass. I initially looked at a number of repair and replicative polymerases. Kf exo$^+$, Kf exo$^-$, pol ε and yeast pol δ were completely blocked by the presence of these adducts. These adducts also posed a significant block to mammalian pol δ. Pol β was able to bypass these adducts only when provided with a phosphorylated gapped substrate, albeit at concentrations when full-length product formation and strand displacement had already occurred on the control substrate. Therefore, these data suggest that these adducts cannot be efficiently bypassed by these polymerases. In addition to testing purified polymerases, I also examined replication past these adducts with a nuclear extract. There was no polymerase in the nuclear extract that was capable of bypassing these adducts, thus suggesting that there is no specific polymerase responsible for the recognition and bypass of these adducts. However, the use of the short adducted oligodeoxynucleotide sequence in the assay may

be a limiting factor in terms of recruitment of PCNA and subsequent polymerases to the stalled replication substrate.

In the search for a polymerase that might be responsible for the bypass of these adducts *in vivo*, I turned my focus to the translesion DNA polymerases that are well suited for replicating past various distorting lesions. Among the various translesion DNA polymerases examined, pol η was found to be efficient in incorporating opposite the adducts, although this process requires several encounters with the polymerase as evidenced through trapping experiments. The nucleotides incorporated by pol η were similar to those incorporated opposite the adducts during replication in mammalian cells, thus providing further evidence that pol η could be involved in the *in vivo* incorporation opposite these adducts. Pol η's capacity to extend past the adducts was significant, although this process also requires more than one encounter with the polymerase. This led to a search for another polymerase that could extend from the incorporation made by pol η. Pol ζ was identified as another ideal candidate for extension as known by its role in extension from mismatched termini and incorporations opposite various adducts (*reviewed in* Prakash et al., 2005). Additionally a synergistic effect between pol η and pol ζ in the bypass of the adducts was observed.

This work also tried to identify a repair mechanism for these adducts through the use of glycosylases that are the initial enzymes in the BER pathway. While none of the glycosylases used (UDG, SMUG1 and Endo III) yielded a positive result, it cannot be ruled out that there exists a potential glycosylase that could excise these adducts. Additionally, NER could also be involved in the removal of these adducts.

The other three DNA adducts presented in this work include the HNE-dG, β-HOEdG and the α*R*- and α*S*-Me-γ-HOPdG adducts. Although HNE-dG adducts displayed a lower mutagenic potential as compared to the BD N3-dU adducts, they exhibited a difference in their mutagenicity among the various stereoisomers. The (*6R, 8S, 11R*) and (*6S, 8R, 11S*) HNE-dG adducts induced at least 4-fold greater point mutations than the (*6R, 8S, 11S*) and (*6S, 8R, 11R*) stereoisomers. Additionally melting studies revealed that the most mutagenic isomers were also the most destabilizing indicating that distortion of the lesion corresponds to its mutagenicity (Wang et al., 2003). The β-HOEdG adducts had a very low mutation yield of 2% and the α*R*- and α*S*-Me-γ-HOPdG adducts had low mutation yields of 4.7 and 6.2 % respectively.

Thus in summary, the DNA adducts presented in this work were all mutagenic and contribute to the overall mutagenicity associated with their parent chemicals.

REFERENCES

Abdel-Rahman, S.Z., Ammenheuser, M.M. and Ward, J.B., Jr. (2001) Human sensitivity to 1,3-butadiene: role of microsomal epoxide hydrolase polymorphisms. *Carcinogenesis*, **22**, 415-423.

Abdel-Rahman, S.Z., El-Zein, R.A., Ammenheuser, M.M., Yang, Z., Stock, T.H., Morandi, M. and Ward, J.B., Jr. (2003) Variability in human sensitivity to 1,3-butadiene: Influence of the allelic variants of the microsomal epoxide hydrolase gene. *Environ Mol Mutagen*, **41**, 140-146.

Adler, I.D., Kliesch, U., Nylund, L. and Peltonen, K. (1997) In vitro and in vivo mutagenicity of the butadiene metabolites butadiene diolepoxide, butadiene monoepoxide and diepoxybutane. *Mutagenesis*, **12**, 339-345.

Akasaka, S. and Guengerich, F.P. (1999) Mutagenicity of site-specifically located 1,N2-ethenoguanine in Chinese hamster ovary cell chromosomal DNA. *Chem Res Toxicol*, **12**, 501-507.

Alary, J., Bravais, F., Cravedi, J.P., Debrauwer, L., Rao, D. and Bories, G. (1995) Mercapturic acid conjugates as urinary end metabolites of the lipid peroxidation product 4-hydroxy-2-nonenal in the rat. *Chem Res Toxicol*, **8**, 34-39.

Alary, J., Debrauwer, L., Fernandez, Y., Cravedi, J.P., Rao, D. and Bories, G. (1998) 1,4-Dihydroxynonene mercapturic acid, the major end metabolite of exogenous 4-hydroxy-2-nonenal, is a physiological component of rat and human urine. *Chem Res Toxicol*, **11**, 130-135.

Albertini, R., Clewell, H., Himmelstein, M.W., Morinello, E., Olin, S., Preston, J., Scarano, L., Smith, M.T., Swenberg, J., Tice, R. and Travis, C. (2003) The use of non-tumor data in cancer risk assessment: reflections on butadiene, vinyl chloride, and benzene. *Regul Toxicol Pharmacol*, **37**, 105-132.

Alin, P., Danielson, U.H. and Mannervik, B. (1985) 4-Hydroxyalk-2-enals are substrates for glutathione transferase. *FEBS Lett*, **179**, 267-270.

Ames, B.N., Durston, W.E., Yamasaki, E. and Lee, F.D. (1973) Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. *Proc Natl Acad Sci U S A*, **70**, 2281-2285.

Bailly, V., Lauder, S., Prakash, S. and Prakash, L. (1997) Yeast DNA repair proteins Rad6 and Rad18 form a heterodimer that has ubiquitin conjugating, DNA binding, and ATP hydrolytic activities. *J Biol Chem*, **272**, 23360-23365.

Bartsch, H., Nair, J. and Owen, R.W. (1999) Dietary polyunsaturated fatty acids and cancers of the breast and colorectum: emerging evidence for their role as risk modifiers. *Carcinogenesis*, **20**, 2209-2218.

Beaucage, S.L.a.I., R.P. (1992) Advances in the Synthesis of Oligonucleotides by the Phosphoramidite Approach. *Tetrahedron*, **48**, 2223-2311.

Bebenek, K., Tissier, A., Frank, E.G., McDonald, J.P., Prasad, R., Wilson, S.H., Woodgate, R. and Kunkel, T.A. (2001) 5'-Deoxyribose phosphate lyase activity of human DNA polymerase iota in vitro. *Science*, **291**, 2156-2159.

Benamira, M., Singh, U. and Marnett, L.J. (1992) Site-specific frameshift mutagenesis by a propanodeoxyguanosine adduct positioned in the (CpG)4 hot-spot of Salmonella typhimurium hisD3052 carried on an M13 vector. *J. Biol. Chem.*, **267**, 22392-22400.

Bernardini, S., Pelin, K., Peltonen, K., Jarventaus, H., Hirvonen, A., Neagu, C., Sorsa, M. and Norppa, H. (1996) Induction of sister chromatid exchange by 3,4-expoxybutane-1,2-diol in cultured human lymphocytes of different GSTT1 and GSTM1 genotypes. *Mutat Res*, **361**, 121-127.

Bolt, H.M. (1988) Roles of etheno-DNA adducts in tumorigenicity of olefins. *Crit Rev Toxicol*, **18**, 299-309.

Bolt, H.M. (1994) Vinyl halides, haloaldehydes and monohaloalkanes. *IARC Sci Publ*, 141-150.

Bond, J.A. and Medinsky, M.A. (2001) Insights into the toxicokinetics and toxicodynamics of 1,3-butadiene. *Chem Biol Interact*, **135-136**, 599-614.

Boogaard, P.J. and Bond, J.A. (1996) The role of hydrolysis in the detoxification of 1,2:3,4-diepoxybutane by human, rat, and mouse liver and lung in vitro. *Toxicol Appl Pharmacol*, **141**, 617-627.

Boogaard, P.J., van Sittert, N.J., Watson, W.P. and de Kloe, K.P. (2001) A novel DNA adduct, originating from 1,2-epoxy-3,4-butanediol, is the major DNA adduct after exposure to [2,3-^{14}C]-1,3-butadiene,[4-^{14}C]-1,2-epoxy-3-butane. *Chem Biol Interact*, **135-136**, 687-693.

Brunnemann, K.D., Kagan, M.R., Cox, J.E. and Hoffmann, D. (1990) Analysis of 1,3-butadiene and other selected gas-phase components in cigarette mainstream and sidestream smoke by gas chromatography-mass selective detection. *Carcinogenesis*, **11**, 1863-1868.

Budiawan and Eder, E. (2000) Detection of 1,N^2-propanodeoxyguanosine adducts in DNA of Fischer 344 rats by an adapted ^{32}P-post-labeling technique after per os application of crotonaldehyde. *Carcinogenesis*, **21**, 1191-1196.

Burczynski, M.E., Sridhar, G.R., Palackal, N.T. and Penning, T.M. (2001) The reactive oxygen species--and Michael acceptor-inducible human aldo-keto reductase AKR1C1 reduces the alpha,beta-unsaturated aldehyde 4-hydroxy-2-nonenal to 1,4-dihydroxy-2-nonene. *J Biol Chem*, **276**, 2890-2897.

Burgers, P.M. (1991) Saccharomyces cerevisiae replication factor C. II. Formation and activity of complexes with the proliferating cell nuclear antigen and with DNA polymerases delta and epsilon. *J Biol Chem*, **266**, 22698-22706.

Cajelli, E., Ferraris, A. and Brambilla, G. (1987) Mutagenicity of 4-hydroxynonenal in V79 Chinese hamster cells. *Mutat Res*, **190**, 169-171.

Carmical, J.R., Kowalczyk, A., Zou, Y., Van Houten, B., Nechev, L.V., Harris, C.M., Harris, T.M. and Lloyd, R.S. (2000a) Butadiene-induced intrastrand DNA cross-links: a possible role in deletion mutagenesis. *J Biol Chem*, **275**, 19482-19489.

Carmical, J.R., Nechev, L.V., Harris, C.M., Harris, T.M. and Lloyd, R.S. (2000b) Mutagenic potential of adenine N^6 adducts of monoepoxide and diolepoxide derivatives of butadiene. *Environ Mol Mutagen*, **35**, 48-56.

Carmical, J.R., Zhang, M., Nechev, L., Harris, C.M., Harris, T.M. and Lloyd, R.S. (2000c) Mutagenic potential of guanine N^2 adducts of butadiene mono- and diolepoxide. *Chem Res Toxicol*, **13**, 18-25.

Casarett, L.J.C., D.K.; Mary, O.A.; Doull J. (1996) *Casarett and Doull's toxicology: the basic science of poisons.*

Chagovetz, A.M., Sweasy, J.B. and Preston, B.D. (1997) Increased activity and fidelity of DNA polymerase beta on single-nucleotide gapped DNA. *J Biol Chem*, **272**, 27501-27504.

Chen, H.J. and Chung, F.L. (1994) Formation of etheno adducts in reactions of enals via autoxidation. *Chem Res Toxicol*, **7**, 857-860.

Cheng, X. and Ruth, J.A. (1993) A simplified methodology for quantitation of butadiene metabolites. Application to the study of 1,3-butadiene metabolism by rat liver microsomes. *Drug Metab Dispos*, **21**, 121-124.

Chung, F.L., Nath, R.G., Nagao, M., Nishikawa, A., Zhou, G.D. and Randerath, K. (1999) Endogenous formation and significance of 1,N^2-propanodeoxyguanosine adducts. *Mutat. Res.*, **424**, 71-81.

Chung, F.L., Nath, R.G., Ocando, J., Nishikawa, A. and Zhang, L. (2000) Deoxyguanosine adducts of t-4-hydroxy-2-nonenal are endogenous DNA lesions in rodents and humans: detection and potential sources. *Cancer Res*, **60**, 1507-1511.

Chung, F.L., Tanaka, T. and Hecht, S.S. (1986) Induction of liver tumors in F344 rats by crotonaldehyde. *Cancer Research*, **46**, 1285-1289.

Ciarrocchi, G. and Pedrini, A.M. (1982) Determination of pyrimidine dimer unwinding angle by measurement of DNA electrophoretic mobility. *J Mol Biol*, **155**, 177-183.

Citti, L., Gervasi, P.G., Turchi, G., Bellucci, G. and Bianchini, R. (1984) The reaction of 3,4-epoxy-1-butene with deoxyguanosine and DNA in vitro: synthesis and characterization of the main adducts. *Carcinogenesis*, **5**, 47-52.

Cochrane, J.E. and Skopek, T.R. (1994a) Mutagenicity of butadiene and its epoxide metabolites: I. Mutagenic potential of 1,2-epoxybutene, 1,2,3,4-diepoxybutane and 3,4-epoxy-1,2-butanediol in cultured human lymphoblasts. *Carcinogenesis*, **15**, 713-717.

Cochrane, J.E. and Skopek, T.R. (1994b) Mutagenicity of butadiene and its epoxide metabolites: II. Mutational spectra of butadiene, 1,2-epoxybutene and diepoxybutane at the hprt locus in splenic T cells from exposed B6C3F1 mice. *Carcinogenesis*, **15**, 719-723.

Cook, J.W. (1937) Chemical compunds as carcinogenic agents. *Am J Cancer*, **29**, 219-259.

Cowles, S.R., Tsai, S.P., Snyder, P.J. and Ross, C.E. (1994) Mortality, morbidity, and haematological results from a cohort of long-term workers involved in 1,3-butadiene monomer production. *Occup Environ Med*, **51**, 323-329.

Csanady, G.A., Guengerich, F.P. and Bond, J.A. (1992) Comparison of the biotransformation of 1,3-butadiene and its metabolite, butadiene monoepoxide, by hepatic and pulmonary tissues from humans, rats and mice. *Carcinogenesis*, **13**, 1143-1153.

Czerny, C., Eder, E. and Runger, T.M. (1998) Genotoxicity and mutagenicity of the alpha, beta-unsaturated carbonyl compound crotonaldehyde (butenal) on a plasmid shuttle vector. *Mutation Research*, **407**, 125-134.

de los Santos, C., Zaliznyak, T. and Johnson, F. (2001) NMR characterization of a DNA duplex containing the major acrolein-derived deoxyguanosine adduct gamma - OH-1,-N2-propano-2'-deoxyguanosine. *J Biol Chem*, **276**, 9077-9082.

Delzell, E., Sathiakumar, N., Hovinga, M., Macaluso, M., Julian, J., Larson, R., Cole, P. and Muir, D.C. (1996) A follow-up study of synthetic rubber workers. *Toxicology*, **113**, 182-189.

Devereux, T.R., Risinger, J.I. and Barrett, J.C. (1999) Mutations and altered expression of the human cancer genes: what they tell us about causes. *IARC Sci Publ*, 19-42.

Dianov, G.L. (2003) Monitoring base excision repair by in vitro assays. *Toxicology*, **193**, 35-41.

Dick, R.A., Kwak, M.K., Sutter, T.R. and Kensler, T.W. (2001) Antioxidative function and substrate specificity of NAD(P)H-dependent alkenal/one oxidoreductase. A new role for leukotriene B4 12-hydroxydehydrogenase/15-oxoprostaglandin 13-reductase. *J Biol Chem*, **276**, 40803-40810.

Divine, B.J. and Hartman, C.M. (1996) Mortality update of butadiene production workers. *Toxicology*, **113**, 169-181.

Divine, B.J., Wendt, J.K. and Hartman, C.M. (1993) Cancer mortality among workers at a butadiene production facility. *IARC Sci Publ*, 345-362.

Downs, T.D., Crane, M.M. and Kim, K.W. (1987) Mortality among workers at a butadiene facility. *Am J Ind Med*, **12**, 311-329.

Duescher, R.J. and Elfarra, A.A. (1994) Human liver microsomes are efficient catalysts of 1,3-butadiene oxidation: evidence for major roles by cytochromes P450 2A6 and 2E1. *Arch Biochem Biophys*, **311**, 342-349.

Eder, E. and Hoffman, C. (1992) Identification and characterization of deoxyguanosine-crotonaldehyde adducts. Formation of 7,8 cyclic adducts and 1,N^2,7,8 bis-cyclic adducts. *Chem Res Toxicol*, **5**, 802-808.

Esposito, G., Godindagger, I., Klein, U., Yaspo, M.L., Cumano, A. and Rajewsky, K. (2000) Disruption of the Rev3l-encoded catalytic subunit of polymerase zeta in mice results in early embryonic lethality. *Curr Biol*, **10**, 1221-1224.

Esterbauer, H., Schaur, R.J. and Zollner, H. (1991) Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med*, **11**, 81-128.

Feng, Z., Hu, W., Amin, S. and Tang, M.S. (2003) Mutational spectrum and genotoxicity of the major lipid peroxidation product, trans-4-hydroxy-2-nonenal, induced DNA adducts in nucleotide excision repair-proficient and -deficient human cells. *Biochemistry*, **42**, 7848-7854.

Fernandes, P.H., Hackfeld, L.C., Kozekov, I.D., Hodge, R.P. and Lloyd, R.S. (2006) Synthesis and Mutagenesis of the Butadiene-Derived N3 2'-Deoxyuridine Adducts. *Chem Res Toxicol*, **19**, 968-976.

Fernandes, P.H., Wang, H., Rizzo, C.J. and Lloyd, R.S. (2003) Site-specific mutagenicity of stereochemically defined 1,N2-deoxyguanosine adducts of trans-4-hydroxynonenal in mammalian cells. *Environ Mol Mutagen*, **42**, 68-74.

Gotz, M.E., Wacker, M., Luckhaus, C., Wanek, P., Tatschner, T., Jellinger, K., Leblhuber, F., Ransmayr, G., Riederer, P. and Eder, E. (2002) Unaltered brain levels of 1,N2-propanodeoxyguanosine adducts of trans-4-hydroxy-2-nonenal in Alzheimer's disease. *Neurosci Lett*, **324**, 49-52.

Graff, J.J., Sathiakumar, N., Macaluso, M., Maldonado, G., Matthews, R. and Delzell, E. (2005) Chemical exposures in the synthetic rubber industry and lymphohematopoietic cancer mortality. *J Occup Environ Med*, **47**, 916-932.

Gros, L., Ishchenko, A.A. and Saparbaev, M. (2003) Enzymology of repair of etheno-adducts. *Mutat Res*, **531**, 219-229.

Haracska, L., Johnson, R.E., Unk, I., Phillips, B.B., Hurwitz, J., Prakash, L. and Prakash, S. (2001a) Targeting of human DNA polymerase iota to the replication machinery via interaction with PCNA. *Proc Natl Acad Sci U S A*, **98**, 14256-14261.

Haracska, L., Prakash, S. and Prakash, L. (2000a) Replication past O^6-methylguanine by yeast and human DNA polymerase eta. *Mol Cell Biol*, **20**, 8001-8007.

Haracska, L., Prakash, S. and Prakash, L. (2003) Yeast DNA polymerase zeta is an efficient extender of primer ends opposite from 7,8-dihydro-8-oxoguanine and O^6-methylguanine. *Mol Cell Biol*, **23**, 1453-1459.

Haracska, L., Unk, I., Johnson, R.E., Johansson, E., Burgers, P.M., Prakash, S. and Prakash, L. (2001b) Roles of yeast DNA polymerases delta and zeta and of Rev1 in the bypass of abasic sites. *Genes Dev*, **15**, 945-954.

Haracska, L., Unk, I., Johnson, R.E., Phillips, B.B., Hurwitz, J., Prakash, L. and Prakash, S. (2002) Stimulation of DNA synthesis activity of human DNA polymerase kappa by PCNA. *Mol Cell Biol*, **22**, 784-791.

Haracska, L., Yu, S.L., Johnson, R.E., Prakash, L. and Prakash, S. (2000b) Efficient and accurate replication in the presence of 7,8-dihydro-8-oxoguanine by DNA polymerase eta. *Nat Genet*, **25**, 458-461.

Hartley, D.P., Ruth, J.A. and Petersen, D.R. (1995) The hepatocellular metabolism of 4-hydroxynonenal by alcohol dehydrogenase, aldehyde dehydrogenase, and glutathione S-transferase. *Arch Biochem Biophys*, **316**, 197-205.

Himmelstein, M.W., Acquavella, J.F., Recio, L., Medinsky, M.A. and Bond, J.A. (1997) Toxicology and epidemiology of 1,3-butadiene. *Crit Rev Toxicol*, **27**, 1-108.

Himmelstein, M.W., Asgharian, B. and Bond, J.A. (1995) High concentrations of butadiene epoxides in livers and lungs of mice compared to rats exposed to 1,3-butadiene. *Toxicol Appl Pharmacol*, **132**, 281-288.

Himmelstein, M.W., Turner, M.J., Asgharian, B. and Bond, J.A. (1994) Comparison of blood concentrations of 1,3-butadiene and butadiene epoxides in mice and rats exposed to 1,3-butadiene by inhalation. *Carcinogenesis*, **15**, 1479-1486.

Hirt, B. (1967) Selective extraction of polyoma DNA from infected mouse cell cultures. *J Mol Biol*, **26**, 365-369.

Hoege, C., Pfander, B., Moldovan, G.L., Pyrowolakis, G. and Jentsch, S. (2002) RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature*, **419**, 135-141.

Hoffmann, J.S., Pillaire, M.J., Maga, G., Podust, V., Hubscher, U. and Villani, G. (1995) DNA polymerase beta bypasses in vitro a single d(GpG)-cisplatin adduct placed on codon 13 of the HRAS gene. *Proc Natl Acad Sci U S A*, **92**, 5356-5360.

http://www.osha.gov/SLTC/butadiene/index.html.

Hu, W., Feng, Z., Eveleigh, J., Iyer, G., Pan, J., Amin, S., Chung, F.L. and Tang, M.S. (2002) The major lipid peroxidation product, trans-4-hydroxy-2-nonenal, preferentially forms DNA adducts at codon 249 of human p53 gene, a unique mutational hotspot in hepatocellular carcinoma. *Carcinogenesis*, **23**, 1781-1789.

Huff, J.E., Melnick, R.L., Solleveld, H.A., Haseman, J.K., Powers, M. and Miller, R.A. (1985) Multiple organ carcinogenicity of 1,3-butadiene in B6C3F1 mice after 60 weeks of inhalation exposure. *Science*, **227**, 548-549.

Hussain, S.P., Raja, K., Amstad, P.A., Sawyer, M., Trudel, L.J., Wogan, G.N., Hofseth, L.J., Shields, P.G., Billiar, T.R., Trautwein, C., Hohler, T., Galle, P.R., Phillips, D.H., Markin, R., Marrogi, A.J. and Harris, C.C. (2000) Increased p53 mutation load in nontumorous human liver of wilson disease and hemochromatosis: oxyradical overload diseases. *Proc Natl Acad Sci U S A*, **97**, 12770-12775.

IARC. (1999) IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, International Agency for Research on Cancer, Lyon. *IARC Sci. Publ.*, **71**, 109-225.

Jackson, M.A., Stack, H.F., Rice, J.M. and Waters, M.D. (2000) A review of the genetic and related effects of 1,3-butadiene in rodents and humans. *Mutat Res*, **463**, 181-213.

Jiang, Y., Zhang, S.J., Wu, S.M. and Lee, M.Y. (1995) Immunoaffinity purification of DNA polymerase delta. *Arch Biochem Biophys*, **320**, 297-304.

Johnson, R.E., Haracska, L., Prakash, S. and Prakash, L. (2001) Role of DNA polymerase zeta in the bypass of a (6-4) TT photoproduct. *Mol Cell Biol*, **21**, 3558-3563.

Johnson, R.E., Kondratick, C.M., Prakash, S. and Prakash, L. (1999a) hRAD30 mutations in the variant form of xeroderma pigmentosum. *Science*, **285**, 263-265.

Johnson, R.E., Prakash, L. and Prakash, S. (2005) Biochemical evidence for the requirement of Hoogsteen base pairing for replication by human DNA polymerase iota. *Proc Natl Acad Sci U S A*, **102**, 10466-10471.

Johnson, R.E., Prakash, S. and Prakash, L. (1999b) Efficient bypass of a thymine-thymine dimer by yeast DNA polymerase, Pol eta. *Science*, **283**, 1001-1004.

Johnson, R.E., Prakash, S. and Prakash, L. (2000a) The human DINB1 gene encodes the DNA polymerase Pol theta. *Proc Natl Acad Sci U S A*, **97**, 3838-3843.

Johnson, R.E., Washington, M.T., Haracska, L., Prakash, S. and Prakash, L. (2000b) Eukaryotic polymerases iota and zeta act sequentially to bypass DNA lesions. *Nature*, **406**, 1015-1019.

Johnson, R.E., Washington, M.T., Prakash, S. and Prakash, L. (2000c) Fidelity of human DNA polymerase eta. *J Biol Chem*, **275**, 7447-7450.

Johnson, R.E., Yu, S.L., Prakash, S. and Prakash, L. (2003) Yeast DNA polymerase zeta (zeta) is essential for error-free replication past thymine glycol. *Genes Dev*, **17**, 77-87.

Kannouche, P., Broughton, B.C., Volker, M., Hanaoka, F., Mullenders, L.H. and Lehmann, A.R. (2001) Domain structure, localization, and function of DNA polymerase eta, defective in xeroderma pigmentosum variant cells. *Genes Dev*, **15**, 158-172.

Kannouche, P., Fernandez de Henestrosa, A.R., Coull, B., Vidal, A.E., Gray, C., Zicha, D., Woodgate, R. and Lehmann, A.R. (2002) Localization of DNA polymerases eta and iota to the replication machinery is tightly co-ordinated in human cells. *EMBO J*, **21**, 6246-6256.

Kannouche, P., Fernandez de Henestrosa, A.R., Coull, B., Vidal, A.E., Gray, C., Zicha, D., Woodgate, R. and Lehmann, A.R. (2003) Localization of DNA polymerases eta and iota to the replication machinery is tightly co-ordinated in human cells. *EMBO J*, **22**, 1223-1233.

Kannouche, P.L. and Lehmann, A.R. (2004) Ubiquitination of PCNA and the polymerase switch in human cells. *Cell Cycle*, **3**, 1011-1013.

Kannouche, P.L., Wing, J. and Lehmann, A.R. (2004) Interaction of human DNA polymerase eta with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage. *Mol Cell*, **14**, 491-500.

Kanuri, M., Minko, I.G., Nechev, L.V., Harris, T.M., Harris, C.M. and Lloyd, R.S. (2002a) Error prone translesion synthesis past gamma-hydroxypropano deoxyguanosine, the primary acrolein-derived adduct in mammalian cells. *J Biol Chem*, **277**, 18257-18265.

Kanuri, M., Nechev, L.V., Tamura, P.J., Harris, C.M., Harris, T.M. and Lloyd, R.S. (2002b) Mutagenic spectrum of butadiene-derived N1-deoxyinosine adducts and N6,N6-deoxyadenosine intrastrand cross-links in mammalian cells. *Chem Res Toxicol*, **15**, 1572-1580.

Karthikeyan, R., Vonarx, E.J., Straffon, A.F., Simon, M., Faye, G. and Kunz, B.A. (2000) Evidence from mutational specificity studies that yeast DNA polymerases delta and epsilon replicate different DNA strands at an intracellular replication fork. *J Mol Biol*, **299**, 405-419.

Kawanishi, M., Matsuda, T., Sasaki, G., Yagi, T., Matsui, S. and Takebe, H. (1998) A spectrum of mutations induced by crotonaldehyde in shuttle vector plasmids propagated in human cells. *Carcinogenesis*, **19**, 69-72.

Kemper, R.A., Elfarra, A.A. and Myers, S.R. (1998) Metabolism of 3-butene-1,2-diol in B6C3F1 mice. Evidence for involvement of alcohol dehydrogenase and cytochrome p450. *Drug Metab Dispos*, **26**, 914-920.

Kennaway, E.L. and Hieger, I. (1930) Carcinogenic susbtances and their fluorescence spectra. *Br Med J*, **1**, 1044-1046.

Kesti, T., Frantti, H. and Syvaoja, J.E. (1993) Molecular cloning of the cDNA for the catalytic subunit of human DNA polymerase epsilon. *J Biol Chem*, **268**, 10238-10245.

Kim, Y., Hong, H.H., Lachat, Y., Clayton, N.P., Devereux, T.R., Melnick, R.L., Hegi, M.E. and Sills, R.C. (2005) Genetic alterations in brain tumors following 1,3-butadiene exposure in B6C3F1 mice. *Toxicol Pathol*, **33**, 307-312.

Klenow, H. and Overgaard-Hansen, K. (1970) Proteolytic cleavage of DNA polymerase from Escherichia Coli B into an exonuclease unit and a polymerase unit. *FEBS Lett*, **6**, 25-27.

Klungland, A. and Lindahl, T. (1997) Second pathway for completion of human DNA base excision-repair: reconstitution with purified proteins and requirement for DNase IV (FEN1). *EMBO J*, **16**, 3341-3348.

Koivisto, P., Adler, I.D., Pacchierotti, F. and Peltonen, K. (1998) DNA adducts in mouse testis and lung after inhalation exposure to 1,3-butadiene. *Mutat Res*, **397**, 3-10.

Koivisto, P., Adler, I.D., Sorsa, M. and Peltonen, K. (1996) Inhalation exposure of rats and mice to 1,3-butadiene induces N6-adenine adducts of epoxybutene detected by 32P-postlabeling and HPLC. *Environ Health Perspect*, **104 Suppl 3**, 655-657.

Kornberg, A., Kornberg, S.R. and Simms, E.S. (1956) Metaphosphate synthesis by an enzyme from Escherichia coli. *Biochim Biophys Acta*, **20**, 215-227.

Kozekov, I.D., Nechev, L.V., Moseley, M.S., Harris, C.M., Rizzo, C.J., Stone, M.P. and Harris, T.M. (2003) DNA interchain cross-links formed by acrolein and crotonaldehyde. *J. Am. Chem. Soc.*, **125**, 50-61.

Kumar, A., Abbotts, J., Karawya, E.M. and Wilson, S.H. (1990) Identification and properties of the catalytic domain of mammalian DNA polymerase beta. *Biochemistry*, **29**, 7156-7159.

Kurtz, A.J. and Lloyd, R.S. (2003) 1,N2-deoxyguanosine adducts of acrolein, crotonaldehyde, and trans-4-hydroxynonenal cross-link to peptides via Schiff base linkage. *J. Biol. Chem.*, **278**, 5970-5976.

Kusumoto, R., Masutani, C., Iwai, S. and Hanaoka, F. (2002) Translesion synthesis by human DNA polymerase eta across thymine glycol lesions. *Biochemistry*, **41**, 6090-6099.

Langouet, S., Mican, A.N., Muller, M., Fink, S.P., Marnett, L.J., Muhle, S.A. and Guengerich, F.P. (1998) Misincorporation of nucleotides opposite five-membered exocyclic ring guanine derivatives by escherichia coli polymerases in vitro and in vivo: 1,N^2-ethenoguanine, 5,6,7,9-tetrahydro-9-oxoimidazo[1, 2-a]purine, and 5,6,7,9-tetrahydro-7-hydroxy-9-oxoimidazo[1, 2-a]purine. *Biochemistry*, **37**, 5184-5193.

Langouet, S., Muller, M. and Guengerich, F.P. (1997) Misincorporation of dNTPs opposite 1,N2-ethenoguanine and 5,6,7,9-tetrahydro-7-hydroxy-9-oxoimidazo[1,2-a]purine in oligonucleotides by Escherichia coli polymerases I exo$^-$ and II exo$^-$, T7 polymerase exo$^-$, human immunodeficiency virus-1 reverse transcriptase, and rat polymerase beta. *Biochemistry*, **36**, 6069-6079.

Lawley, P.D. and Brookes, P. (1967) Interstrand cross-linking of DNA by difunctional alkylating agents. *J Mol Biol*, **25**, 143-160.

Lee, M.Y., Tan, C.K., Downey, K.M. and So, A.G. (1984) Further studies on calf thymus DNA polymerase delta purified to homogeneity by a new procedure. *Biochemistry*, **23**, 1906-1913.

Lehman, I.R., Bessman, M.J., Simms, E.S. and Kornberg, A. (1958) Enzymatic synthesis of deoxyribonucleic acid. I. Preparation of substrates and partial purification of an enzyme from Escherichia coli. *J Biol Chem*, **233**, 163-170.

Lehmann, A.R. (2005) Replication of damaged DNA by translesion synthesis in human cells. *FEBS Lett*, **579**, 873-876.

Lehmann, M.B.A. (2001) *Advances in Genetics*. Academic Press, San Diego.

Leuratti, C., Jones, N.J., Marafante, E., Kostiainen, R., Peltonen, K. and Waters, R. (1994) DNA damage induced by the environmental carcinogen butadiene: identification of a diepoxybutane-adenine adduct and its detection by ^{32}P-postlabelling. *Carcinogenesis*, **15**, 1903-1910.

Li, F., Segal, A. and Solomon, J.J. (1992) In vitro reaction of ethylene oxide with DNA and characterization of DNA adducts. *Chem Biol Interact*, **83**, 35-54.

Lu, C.D. and Byrnes, J.J. (1992) PCNA-dependent DNA polymerase delta from rabbit bone marrow. *Biochemistry*, **31**, 12403-12409.

Macaluso, M., Larson, R., Delzell, E., Sathiakumar, N., Hovinga, M., Julian, J., Muir, D. and Cole, P. (1996) Leukemia and cumulative exposure to butadiene, styrene and benzene among workers in the synthetic rubber industry. *Toxicology*, **113**, 190-202.

Maher, V.M., Ouellette, L.M., Curren, R.D. and McCormick, J.J. (1976) Frequency of ultraviolet light-induced mutations is higher in xeroderma pigmentosum variant cells than in normal human cells. *Nature*, **261**, 593-595.

Malvoisin, E., Evrard, E., Roberfroid, M. and Mercier, M. (1979) Determination of Kovats retention indices with a capillary column and electron-capture detection: application to the assay of the enzymatic conversion of 3,4-epoxy-1-butene into diepoxybutane. *J Chromatogr*, **186**, 81-87.

Malvoisin, E. and Roberfroid, M. (1982) Hepatic microsomal metabolism of 1,3-butadiene. *Xenobiotica*, **12**, 137-144.

Marnett, L.J. (1994) DNA adducts of alpha,beta-unsaturated aldehydes and dicarbonyl compounds. *IARC Sci Publ*, 151-163.

Marnett, L.J. (1999) Chemistry and biology of DNA damage by malondialdehyde. *IARC Sci Publ*, 17-27.

Martinelli, M.J., Vaidyanathan, R., Pawlak, J.M., Nayyar, N.K., Dhokte, U.P., Doecke, C.W., Zollars, L.M., Moher, E.D., Khau, V.V. and Kosmrlj, B. (2002) Catalytic regioselective sulfonylation of alpha-chelatable alcohols: scope and mechanistic insight. *J Am Chem Soc*, **124**, 3578-3585.

Masutani, C., Araki, M., Yamada, A., Kusumoto, R., Nogimori, T., Maekawa, T., Iwai, S. and Hanaoka, F. (1999a) Xeroderma pigmentosum variant (XP-V) correcting protein from HeLa cells has a thymine dimer bypass DNA polymerase activity. *EMBO J*, **18**, 3491-3501.

Masutani, C., Kusumoto, R., Iwai, S. and Hanaoka, F. (2000) Mechanisms of accurate translesion synthesis by human DNA polymerase eta. *EMBO J*, **19**, 3100-3109.

Masutani, C., Kusumoto, R., Yamada, A., Dohmae, N., Yokoi, M., Yuasa, M., Araki, M., Iwai, S., Takio, K. and Hanaoka, F. (1999b) The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase eta. *Nature*, **399**, 700-704.

Matanoski, G., Francis, M., Correa-Villasenor, A., Elliott, E., Santos-Burgoa, C. and Schwartz, L. (1993) Cancer epidemiology among styrene-butadiene rubber workers. *IARC Sci Publ*, 363-374.

Matsuda, T., Bebenek, K., Masutani, C., Hanaoka, F. and Kunkel, T.A. (2000) Low fidelity DNA synthesis by human DNA polymerase-eta. *Nature*, **404**, 1011-1013.

Matsumoto, Y. and Bogenhagen, D.F. (1991) Repair of a synthetic abasic site involves concerted reactions of DNA synthesis followed by excision and ligation. *Mol Cell Biol*, **11**, 4441-4447.

Matsumoto, Y. and Kim, K. (1995) Excision of deoxyribose phosphate residues by DNA polymerase beta during DNA repair. *Science*, **269**, 699-702.

Matsumoto, Y., Kim, K., Hurwitz, J., Gary, R., Levin, D.S., Tomkinson, A.E. and Park, M.S. (1999) Reconstitution of proliferating cell nuclear antigen-dependent repair of apurinic/apyrimidinic sites with purified human proteins. *J Biol Chem*, **274**, 33703-33708.

McDonald, J.P., Rapic-Otrin, V., Epstein, J.A., Broughton, B.C., Wang, X., Lehmann, A.R., Wolgemuth, D.J. and Woodgate, R. (1999) Novel human and mouse homologs of Saccharomyces cerevisiae DNA polymerase eta. *Genomics*, **60**, 20-30.

McLaughlin, J.K. and Lipworth, L. (1999) A critical review of the epidemiologic literature on health effects of occupational exposure to vinyl chloride. *J Epidemiol Biostat*, **4**, 253-275.

Meinhardt, T.J., Lemen, R.A., Crandall, M.S. and Young, R.J. (1982) Environmental epidemiologic investigation of the styrene-butadiene rubber industry. Mortality

patterns with discussion of the hematopoietic and lymphatic malignancies. *Scand J Work Environ Health*, **8**, 250-259.

Melnick, R.L., Huff, J., Chou, B.J. and Miller, R.A. (1990) Carcinogenicity of 1,3-butadiene in C57BL/6 x C3H F1 mice at low exposure concentrations. *Cancer Res*, **50**, 6592-6599.

Merritt, W.K., Kowalczyk, A., Scholdberg, T.A., Dean, S.M., Harris, T.M., Harris, C.M., Lloyd, R.S. and Stone, M.P. (2005) Dual roles of glycosyl torsion angle conformation and stereochemical configuration in butadiene oxide-derived N1 beta-hydroxyalkyl deoxyinosine adducts: a structural perspective. *Chem Res Toxicol*, **18**, 1098-1107.

Millard, J.T. and White, M.M. (1993) Diepoxybutane cross-links DNA at 5'-GNC sequences. *Biochemistry*, **32**, 2120-2124.

Miller, E.C. and Miller, J.A. (1947) The presence and significance of bound amino azodyes in the livers of rats fed p -dimethylaminoazobenzene. *Cancer Res*, **7**, 468-480.

Miller, E.C. and Miller, J.A. (1979) Milestones in chemical carcinogenesis. *Semin Oncol*, **6**, 445-460.

Minko, I.G., Washington, M.T., Kanuri, M., Prakash, L., Prakash, S. and Lloyd, R.S. (2003) Translesion synthesis past acrolein-derived DNA adduct, gamma -hydroxypropanodeoxyguanosine, by yeast and human DNA polymerase eta. *J Biol Chem*, **278**, 784-790.

Mitchell, D.Y. and Petersen, D.R. (1987) The oxidation of alpha-beta unsaturated aldehydic products of lipid peroxidation by rat liver aldehyde dehydrogenases. *Toxicol Appl Pharmacol*, **87**, 403-410.

Moriya, M. (1993) Single-stranded shuttle phagemid for mutagenesis studies in mammalian cells: 8-oxoguanine in DNA induces targeted G.C-->T.A transversions in simian kidney cells. *Proc Natl Acad Sci U S A*, **90**, 1122-1126.

Moriya, M., Zhang, W., Johnson, F. and Grollman, A.P. (1994) Mutagenic potency of exocyclic DNA adducts: marked differences between Escherichia coli and simian kidney cells. *Proc Natl Acad Sci U S A*, **91**, 11899-11903.

Nair, D.T., Johnson, R.E., Prakash, L., Prakash, S. and Aggarwal, A.K. (2005) Human DNA polymerase iota incorporates dCTP opposite template G via a G.C + Hoogsteen base pair. *Structure*, **13**, 1569-1577.

Napoli, C., D'Armiento, F.P., Mancini, F.P., Postiglione, A., Witztum, J.L., Palumbo, G. and Palinski, W. (1997) Fatty streak formation occurs in human fetal aortas and is greatly enhanced by maternal hypercholesterolemia. Intimal accumulation of low density lipoprotein and its oxidation precede monocyte recruitment into early atherosclerotic lesions. *J Clin Invest*, **100**, 2680-2690.

National Toxicology Program, US Dept of Health and Human Services, Washington, DC. (2001) The Ninth Report on Carcinogens.

Nauhaus, S.K., Fennell, T.R., Asgharian, B., Bond, J.A. and Sumner, S.C. (1996) Characterization of urinary metabolites from Sprague-Dawley rats and B6C3F1 mice exposed to [1,2,3,4-^{13}C]butadiene. *Chem Res Toxicol*, **9**, 764-773.

Neagu, I., Koivisto, P., Neagu, C., Kaltia, S., Kostiainen, R. and Peltonen, K. (1994) Alkylation of purine bases with 3,4-epoxy-1-butene, a reactive metabolite of 1,3-butadiene. *IARC Sci Publ*, 419-422.

Nechev, L.V., Harris, C.M. and Harris, T.M. (2000) Synthesis of nucleosides and oligonucleotides containing adducts of acrolein and vinyl chloride. *Chem Res Toxicol*, **13**, 421-429.

Nechev, L.V., Kozekov, I., Harris, C.M. and Harris, T.M. (2001) Stereospecific synthesis of oligonucleotides containing crotonaldehyde adducts of deoxyguanosine. *Chem Res Toxicol*, **14**, 1506-1512.

Nelson, J.R., Lawrence, C.W. and Hinkle, D.C. (1996a) Deoxycytidyl transferase activity of yeast REV1 protein. *Nature*, **382**, 729-731.

Nelson, J.R., Lawrence, C.W. and Hinkle, D.C. (1996b) Thymine-thymine dimer bypass by yeast DNA polymerase zeta. *Science*, **272**, 1646-1649.

O'Connor, T., Ireland, L.S., Harrison, D.J. and Hayes, J.D. (1999) Major differences exist in the function and tissue-specific expression of human aflatoxin B1 aldehyde reductase and the principal human aldo-keto reductase AKR1 family members. *Biochem J*, **343 Pt 2**, 487-504.

O'Day, C.L., Burgers, P.M. and Taylor, J.S. (1992) PCNA-induced DNA synthesis past cis-syn and trans-syn-I thymine dimers by calf thymus DNA polymerase delta in vitro. *Nucleic Acids Res*, **20**, 5403-5406.

Ogi, T., Kannouche, P. and Lehmann, A.R. (2005) Localisation of human Y-family DNA polymerase kappa: relationship to PCNA foci. *J Cell Sci*, **118**, 129-136.

Ogi, T., Kato, T., Jr., Kato, T. and Ohmori, H. (1999) Mutation enhancement by DINB1, a mammalian homologue of the Escherichia coli mutagenesis protein dinB. *Genes Cells*, **4**, 607-618.

Ogi, T., Mimura, J., Hikida, M., Fujimoto, H., Fujii-Kuriyama, Y. and Ohmori, H. (2001) Expression of human and mouse genes encoding pol kappa: testis-specific developmental regulation and AhR-dependent inducible transcription. *Genes Cells*, **6**, 943-953.

Ogi, T., Shinkai, Y., Tanaka, K. and Ohmori, H. (2002) Pol kappa protects mammalian cells against the lethal and mutagenic effects of benzo[a]pyrene. *Proc Natl Acad Sci U S A*, **99**, 15548-15553.

Ohashi, E., Bebenek, K., Matsuda, T., Feaver, W.J., Gerlach, V.L., Friedberg, E.C., Ohmori, H. and Kunkel, T.A. (2000) Fidelity and processivity of DNA synthesis by DNA polymerase kappa, the product of the human DINB1 gene. *J Biol Chem*, **275**, 39678-39684.

Ollis, D.L., Brick, P., Hamlin, R., Xuong, N.G. and Steitz, T.A. (1985) Structure of large fragment of Escherichia coli DNA polymerase I complexed with dTMP. *Nature*, **313**, 762-766.

Osterman-Golkar, S., Kautiainen, A., Bergmark, E., Hakansson, K. and Maki-Paakkanen, J. (1991) Hemoglobin adducts and urinary mercapturic acids in rats as biological indicators of butadiene exposure. *Chem Biol Interact*, **80**, 291-302.

Osterman-Golkar, S.M., Bond, J.A., Ward, J.B., Jr. and Legator, M.S. (1993) Use of haemoglobin adducts for biomonitoring exposure to 1,3-butadiene. *IARC Sci Publ*, 127-134.

Owen, P.E. and Glaister, J.R. (1990) Inhalation toxicity and carcinogenicity of 1,3-butadiene in Sprague-Dawley rats. *Environ Health Perspect*, **86**, 19-25.

Owen, P.E., Glaister, J.R., Gaunt, I.F. and Pullinger, D.H. (1987) Inhalation toxicity studies with 1,3-butadiene. 3. Two year toxicity/carcinogenicity study in rats. *Am Ind Hyg Assoc J*, **48**, 407-413.

Pan, J. and Chung, F.L. (2002) Formation of cyclic deoxyguanosine adducts from omega-3 and omega-6 polyunsaturated fatty acids under oxidative conditions. *Chem Res Toxicol*, **15**, 367-372.

Park, S., Hodge, J., Anderson, C. and Tretyakova, N. (2004) Guanine-adenine DNA cross-linking by 1,2,3,4-diepoxybutane: potential basis for biological activity. *Chem Res Toxicol*, **17**, 1638-1651.

Park, S. and Tretyakova, N. (2004) Structural characterization of the major DNA-DNA cross-link of 1,2,3,4-diepoxybutane. *Chem Res Toxicol*, **17**, 129-136.

Parola, M., Bellomo, G., Robino, G., Barrera, G. and Dianzani, M.U. (1999) 4-Hydroxynonenal as a biological signal: molecular basis and pathophysiological implications. *Antioxid Redox Signal*, **1**, 255-284.

Pascucci, B., Stucki, M., Jonsson, Z.O., Dogliotti, E. and Hubscher, U. (1999) Long patch base excision repair with purified human proteins. DNA ligase I as patch size mediator for DNA polymerases delta and epsilon. *J Biol Chem*, **274**, 33696-33702.

Pavlov, Y.I., Shcherbakova, P.V. and Kunkel, T.A. (2001) In vivo consequences of putative active site mutations in yeast DNA polymerases alpha, epsilon, delta, and zeta. *Genetics*, **159**, 47-64.

Pelz, N., Dempster, N.M. and Shore, P.R. (1990) Analysis of low molecular weight hydrocarbons including 1,3-butadiene in engine exhaust gases using an aluminum oxide porous-layer open-tubular fused-silica column. *J Chromatogr Sci*, **28**, 230-235.

Perez, H.L., Lahdetie, J., Landin, H., Kilpelainen, I., Koivisto, P., Peltonen, K. and Osterman-Golkar, S. (1997) Haemoglobin adducts of epoxybutanediol from exposure to 1,3-butadiene or butadiene epoxides. *Chem Biol Interact*, **105**, 181-198.

Petersen, D.R. and Doorn, J.A. (2004) Reactions of 4-hydroxynonenal with proteins and cellular targets. *Free Radic Biol Med*, **37**, 937-945.

Plna, K., Nilsson, R., Koskinen, M. and Segerback, D. (1999) ^{32}P-postlabelling of propylene oxide 1- and N^6-substituted adenine and 3-substituted cytosine/uracil: formation and persistence in vitro and in vivo. *Carcinogenesis*, **20**, 2025-2032.

Plug, A.W., Clairmont, C.A., Sapi, E., Ashley, T. and Sweasy, J.B. (1997) Evidence for a role for DNA polymerase beta in mammalian meiosis. *Proc Natl Acad Sci U S A*, **94**, 1327-1331.

Podlutsky, A.J., Dianova, II, Podust, V.N., Bohr, V.A. and Dianov, G.L. (2001) Human DNA polymerase beta initiates DNA synthesis during long-patch repair of reduced AP sites in DNA. *EMBO J*, **20**, 1477-1482.

Poli, G. and Schaur, R.J. (2000) 4-Hydroxynonenal in the pathomechanisms of oxidative stress. *IUBMB Life*, **50**, 315-321.

Pott, P. (1963) Chirurgical observations relative to the cancer of the scrotum. *Natl Cancer Inst Monograph*, **10**, 7-13.

Powley, M.W., Jayaraj, K., Gold, A., Ball, L.M. and Swenberg, J.A. (2003) 1,N2-propanodeoxyguanosine adducts of the 1,3-butadiene metabolite, hydroxymethylvinyl ketone. *Chem Res Toxicol*, **16**, 1448-1454.

Powley, M.W., Li, Y., Upton, P.B., Walker, V.E. and Swenberg, J.A. (2005) Quantification of DNA and hemoglobin adducts of 3,4-epoxy-1,2-butanediol in rodents exposed to 3-butene-1,2-diol. *Carcinogenesis*, **26**, 1573-1580.

Prakash, S., Johnson, R.E. and Prakash, L. (2005) Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function. *Annu Rev Biochem*, **74**, 317-353.

Prakash, S. and Prakash, L. (2002) Translesion DNA synthesis in eukaryotes: a one- or two-polymerase affair. *Genes Dev*, **16**, 1872-1883.

Prasad, R., Beard, W.A. and Wilson, S.H. (1994) Studies of gapped DNA substrate binding by mammalian DNA polymerase beta. Dependence on 5'-phosphate group. *J Biol Chem*, **269**, 18096-18101.

Prelich, G., Tan, C.K., Kostura, M., Mathews, M.B., So, A.G., Downey, K.M. and Stillman, B. (1987) Functional identity of proliferating cell nuclear antigen and a DNA polymerase-delta auxiliary protein. *Nature*, **326**, 517-520.

Purchase, I.F., Stafford, J. and Paddle, G.M. (1987) Vinyl chloride: an assessment of the risk of occupational exposure. *Food Chem Toxicol*, **25**, 187-202.

Roche, H., Gietz, R.D. and Kunz, B.A. (1994) Specificity of the yeast rev3 delta antimutator and REV3 dependency of the mutator resulting from a defect (rad1 delta) in nucleotide excision repair. *Genetics*, **137**, 637-646.

Sanchez, A.M., Minko, I.G., Kurtz, A.J., Kanuri, M., Moriya, M. and Lloyd, R.S. (2003) Comparative evaluation of the bioreactivity and mutagenic spectra of acrolein-derived alpha-HOPdG and gamma-HOPdG regioisomeric deoxyguanosine adducts. *Chem Res Toxicol*, **16**, 1019-1028.

Santos-Burgoa, C., Matanoski, G.M., Zeger, S. and Schwartz, L. (1992) Lymphohematopoietic cancer in styrene-butadiene polymerization workers. *Am J Epidemiol*, **136**, 843-854.

Sarkar, S., Davies, A.A., Ulrich, H.D. and McHugh, P.J. (2006) DNA interstrand crosslink repair during G1 involves nucleotide excision repair and DNA polymerase zeta. *EMBO J*, **25**, 1285-1294.

Sayre, L.M., Zelasko, D.A., Harris, P.L., Perry, G., Salomon, R.G. and Smith, M.A. (1997) 4-Hydroxynonenal-derived advanced lipid peroxidation end products are increased in Alzheimer's disease. *J Neurochem*, **68**, 2092-2097.

Schneider, C., Tallman, K.A., Porter, N.A. and Brash, A.R. (2001) Two distinct pathways of formation of 4-hydroxynonenal. Mechanisms of nonenzymatic transformation of the 9- and 13-hydroperoxides of linoleic acid to 4-hydroxyalkenals. *J Biol Chem*, **276**, 20831-20838.

Scholdberg, T.A., Merritt, W.K., Dean, S.M., Kowalcyzk, A., Harris, C.M., Harris, T.M., Rizzo, C.J., Lloyd, R.S. and Stone, M.P. (2005) Structure of an oligodeoxynucleotide containing a butadiene oxide-derived N1 beta-hydroxyalkyl deoxyinosine adduct in the human N-ras codon 61 sequence. *Biochemistry*, **44**, 3327-3337.

Seaton, M.J., Follansbee, M.H. and Bond, J.A. (1995) Oxidation of 1,2-epoxy-3-butene to 1,2:3,4-diepoxybutane by cDNA-expressed human cytochromes P450 2E1 and 3A4 and human, mouse and rat liver microsomes. *Carcinogenesis*, **16**, 2287-2293.

Segal, A., Solomon, J.J. and Mukai, F. (1990) In vitro reactions of glycidol with pyrimidine bases in calf thymus DNA. *Cancer Biochem Biophys*, **11**, 59-67.

Selzer, R.R. and Elfarra, A.A. (1996a) Characterization of N1- and N^6-adenosine adducts and N1-inosine adducts formed by the reaction of butadiene monoxide with adenosine: evidence for the N1-adenosine adducts as major initial products. *Chem Res Toxicol*, **9**, 875-881.

Selzer, R.R. and Elfarra, A.A. (1996b) Synthesis and biochemical characterization of N1-, N^2-, and N7-guanosine adducts of butadiene monoxide. *Chem Res Toxicol*, **9**, 126-132.

Selzer, R.R. and Elfarra, A.A. (1997a) Characterization of four N-3-thymidine adducts formed in vitro by the reaction of thymidine and butadiene monoxide. *Carcinogenesis*, **18**, 1993-1998.

Selzer, R.R. and Elfarra, A.A. (1997b) Chemical modification of deoxycytidine at different sites yields adducts of different stabilities: characterization of N3- and O2-deoxycytidine and N3-deoxyuridine adducts of butadiene monoxide. *Arch Biochem Biophys*, **343**, 63-72.

Selzer, R.R. and Elfarra, A.A. (1999) In vitro reactions of butadiene monoxide with single- and double-stranded DNA: characterization and quantitation of several purine and pyrimidine adducts. *Carcinogenesis*, **20**, 285-292.

Servant, L., Cazaux, C., Bieth, A., Iwai, S., Hanaoka, F. and Hoffmann, J.S. (2002) A role for DNA polymerase beta in mutagenic UV lesion bypass. *J Biol Chem*, **277**, 50046-50053.

Shcherbakova, P.V., Bebenek, K. and Kunkel, T.A. (2003) Functions of eukaryotic DNA polymerases. *Sci Aging Knowledge Environ*, **2003**, RE3.

Shcherbakova, P.V., Noskov, V.N., Pshenichnov, M.R. and Pavlov, Y.I. (1996) Base analog 6-N-hydroxylaminopurine mutagenesis in the yeast Saccharomyces cerevisiae is controlled by replicative DNA polymerases. *Mutat Res*, **369**, 33-44.

Shcherbakova, P.V. and Pavlov, Y.I. (1996) 3'-->5' exonucleases of DNA polymerases epsilon and delta correct base analog induced DNA replication errors on opposite DNA strands in Saccharomyces cerevisiae. *Genetics*, **142**, 717-726.

Shibutani, S., Suzuki, N. and Grollman, A.P. (1998) Mutagenic specificity of (acetylamino)fluorene-derived DNA adducts in mammalian cells. *Biochemistry*, **37**, 12034-12041.

Shimizu, K., Hashimoto, K., Kirchner, J.M., Nakai, W., Nishikawa, H., Resnick, M.A. and Sugino, A. (2002) Fidelity of DNA polymerase epsilon holoenzyme from budding yeast Saccharomyces cerevisiae. *J Biol Chem*, **277**, 37422-37429.

Singer, B., Medina, M., Zhang, Y., Wang, Z., Guliaev, A.B. and Hang, B. (2002) 8-(Hydroxymethyl)-3,N(4)-etheno-C, a potential carcinogenic glycidaldehyde product, miscodes in vitro using mammalian DNA polymerases. *Biochemistry*, **41**, 1778-1785.

Singh, U.S., Decker-Samuelian, K. and Solomon, J.J. (1996) Reaction of epichlorohydrin with 2'-deoxynucleosides: characterization of adducts. *Chem Biol Interact*, **99**, 109-128.

Singhal, R.K. and Wilson, S.H. (1993) Short gap-filling synthesis by DNA polymerase beta is processive. *J Biol Chem*, **268**, 15906-15911.

Sodum, R.S. and Chung, F.L. (1989) Structural characterization of adducts formed in the reaction of 2,3-epoxy-4-hydroxynonanal with deoxyguanosine. *Chem Res Toxicol*, **2**, 23-28.

Solomon, J.J., Mukai, F., Fedyk, J. and Segal, A. (1988) Reactions of propylene oxide with 2'-deoxynucleosides and in vitro with calf thymus DNA. *Chem Biol Interact*, **67**, 275-294.

Solomon, J.J. and Segal, A. (1989) DNA adducts of propylene oxide and acrylonitrile epoxide: hydrolytic deamination of 3-alkyl-dCyd to 3-alkyl-dUrd. *Environ Health Perspect*, **81**, 19-22.

Solomon, J.J., Singh, U.S. and Segal, A. (1993) In vitro reactions of 2-cyanoethylene oxide with calf thymus DNA. *Chem Biol Interact*, **88**, 115-135.

Srivastava, S., Watowich, S.J., Petrash, J.M., Srivastava, S.K. and Bhatnagar, A. (1999) Structural and kinetic determinants of aldehyde reduction by aldose reductase. *Biochemistry*, **38**, 42-54.

Steitz, T.A., Smerdon, S.J., Jager, J. and Joyce, C.M. (1994) A unified polymerase mechanism for nonhomologous DNA and RNA polymerases. *Science*, **266**, 2022-2025.

Stucki, M., Pascucci, B., Parlanti, E., Fortini, P., Wilson, S.H., Hubscher, U. and Dogliotti, E. (1998) Mammalian base excision repair by DNA polymerases delta and epsilon. *Oncogene*, **17**, 835-843.

Sugo, N., Aratani, Y., Nagashima, Y., Kubota, Y. and Koyama, H. (2000) Neonatal lethality with abnormal neurogenesis in mice deficient in DNA polymerase beta. *Embo J*, **19**, 1397-1404.

Syvaoja, J., Suomensaari, S., Nishida, C., Goldsmith, J.S., Chui, G.S., Jain, S. and Linn, S. (1990) DNA polymerases alpha, delta, and epsilon: three distinct enzymes from HeLa cells. *Proc Natl Acad Sci U S A*, **87**, 6664-6668.

Tan, C.K., Castillo, C., So, A.G. and Downey, K.M. (1986) An auxiliary protein for DNA polymerase-delta from fetal calf thymus. *J Biol Chem*, **261**, 12310-12316.

Thomas, D.C., Roberts, J.D., Sabatino, R.D., Myers, T.W., Tan, C.K., Downey, K.M., So, A.G., Bambara, R.A. and Kunkel, T.A. (1991) Fidelity of mammalian DNA replication and replicative DNA polymerases. *Biochemistry*, **30**, 11751-11759.

Tissier, A., Frank, E.G., McDonald, J.P., Iwai, S., Hanaoka, F. and Woodgate, R. (2000a) Misinsertion and bypass of thymine-thymine dimers by human DNA polymerase iota. *EMBO J*, **19**, 5259-5266.

Tissier, A., McDonald, J.P., Frank, E.G. and Woodgate, R. (2000b) pol iota, a remarkably error-prone human DNA polymerase. *Genes Dev*, **14**, 1642-1650.

Tretyakova, N., Chiang, S.Y., Walker, V.E. and Swenberg, J.A. (1998) Quantitative analysis of 1,3-butadiene-induced DNA adducts in vivo and in vitro using liquid chromatography electrospray ionization tandem mass spectrometry. *J Mass Spectrom*, **33**, 363-376.

Tretyakova, N., Lin, Y., Sangaiah, R., Upton, P.B. and Swenberg, J.A. (1997) Identification and quantitation of DNA adducts from calf thymus DNA exposed to 3,4-epoxy-1-butene. *Carcinogenesis*, **18**, 137-147.

Tretyakova, N., Lin, Y.P., Upton, P.B., Sangaiah, R. and Swenberg, J.A. (1996) Macromolecular adducts of butadiene. *Toxicology*, **113**, 70-76.

Tretyakova, N., Matter, B., Ogdie, A., Wishnok, J.S. and Tannenbaum, S.R. (2001) Locating nucleobase lesions within DNA sequences by MALDI-TOF mass spectral analysis of exonuclease ladders. *Chem Res Toxicol*, **14**, 1058-1070.

USEPA. (2002) Health Assessment of 1,3-Butadiene, United States Environmental Protection Agency National Center for Environmental Assessment, Washington DC., **Publication 600/P-98/001F.**

Vaisman, A. and Chaney, S.G. (2000) The efficiency and fidelity of translesion synthesis past cisplatin and oxaliplatin GpG adducts by human DNA polymerase beta. *J Biol Chem*, **275**, 13017-13025.

Vaisman, A., Frank, E.G., Iwai, S., Ohashi, E., Ohmori, H., Hanaoka, F. and Woodgate, R. (2003) Sequence context-dependent replication of DNA templates containing UV-induced lesions by human DNA polymerase iota. *DNA Repair (Amst)*, **2**, 991-1006.

Vaisman, A., Takasawa, K., Iwai, S. and Woodgate, R. (2006) DNA polymerase iota-dependent translesion replication of uracil containing cyclobutane pyrimidine dimers. *DNA Repair (Amst)*, **5**, 210-218.

Vaisman, A. and Woodgate, R. (2001) Unique misinsertion specificity of pol iota may decrease the mutagenic potential of deaminated cytosines. *EMBO J*, **20**, 6520-6529.

Vander Jagt, D.L., Kolb, N.S., Vander Jagt, T.J., Chino, J., Martinez, F.J., Hunsaker, L.A. and Royer, R.E. (1995) Substrate specificity of human aldose reductase: identification of 4-hydroxynonenal as an endogenous substrate. *Biochim Biophys Acta*, **1249**, 117-126.

Vanduuren, B.L., Nelson, N., Orris, L., Palmes, E.D. and Schmitt, F.L. (1963) Carcinogenicity of Epoxides, Lactones, and Peroxy Compounds. *J Natl Cancer Inst*, **31**, 41-55.

Wacker, M., Wanek, P. and Eder, E. (2001) Detection of 1,N^2-propanodeoxyguanosine adducts of trans-4-hydroxy-2-nonenal after gavage of trans-4-hydroxy-2-nonenal or induction of lipid peroxidation with carbon tetrachloride in F344 rats. *Chem Biol Interact*, **137**, 269-283.

Wang, C.I. and Taylor, J.S. (1991) Site-specific effect of thymine dimer formation on dAn.dTn tract bending and its biological implications. *Proc Natl Acad Sci U S A*, **88**, 9072-9076.

Wang, H., Kozekov, I.D., Harris, T.M. and Rizzo, C.J. (2003) Site-specific synthesis and reactivity of oligonucleotides containing stereochemically defined 1,N^2-deoxyguanosine adducts of the lipid peroxidation product trans-4-hydroxynonenal. *J Am Chem Soc*, **125**, 5687-5700.

Wang, H. and Rizzo, C.J. (2001) Stereocontrolled syntheses of all four stereoisomeric 1,N2-deoxyguanosine adducts of the lipid peroxidation product trans-4-hydroxynonenal. *Org Lett*, **3**, 3603-3605.

Ward, J.B., Jr., Ammenheuser, M.M., Whorton, E.B., Jr., Bechtold, W.E., Kelsey, K.T. and Legator, M.S. (1996) Biological monitoring for mutagenic effects of occupational exposure to butadiene. *Toxicology*, **113**, 84-90.

Washington, M.T., Johnson, R.E., Prakash, L. and Prakash, S. (2002) Human DINB1-encoded DNA polymerase kappa is a promiscuous extender of mispaired primer termini. *Proc Natl Acad Sci U S A*, **99**, 1910-1914.

Washington, M.T., Johnson, R.E., Prakash, L. and Prakash, S. (2004a) Human DNA polymerase iota utilizes different nucleotide incorporation mechanisms dependent upon the template base. *Mol Cell Biol*, **24**, 936-943.

Washington, M.T., Johnson, R.E., Prakash, S. and Prakash, L. (1999) Fidelity and processivity of Saccharomyces cerevisiae DNA polymerase eta. *J Biol Chem*, **274**, 36835-36838.

Washington, M.T., Minko, I.G., Johnson, R.E., Haracska, L., Harris, T.M., Lloyd, R.S., Prakash, S. and Prakash, L. (2004b) Efficient and error-free replication past a minor-groove N^2-guanine adduct by the sequential action of yeast Rev1 and DNA polymerase zeta. *Mol Cell Biol*, **24**, 6900-6906.

Washington, M.T., Minko, I.G., Johnson, R.E., Wolfle, W.T., Harris, T.M., Lloyd, R.S., Prakash, S. and Prakash, L. (2004c) Efficient and error-free replication past a minor-groove DNA adduct by the sequential action of human DNA polymerases iota and kappa. *Mol Cell Biol*, **24**, 5687-5693.

Watanabe, K., Tateishi, S., Kawasuji, M., Tsurimoto, T., Inoue, H. and Yamaizumi, M. (2004) Rad18 guides pol eta to replication stalling sites through physical interaction and PCNA monoubiquitination. *EMBO J*, **23**, 3886-3896.

Wickliffe, J.K., Ammenheuser, M.M., Salazar, J.J., Abdel-Rahman, S.Z., Hastings-Smith, D.A., Postlethwait, E.M., Lloyd, R.S. and Ward, J.B., Jr. (2003) A model of sensitivity: 1,3-butadiene increases mutant frequencies and genomic damage in mice lacking a functional microsomal epoxide hydrolase gene. *Environ Mol Mutagen*, **42**, 106-110.

Wickliffe, J.K., Galbert, L.A., Ammenheuser, M.M., Herring, S.M., Xie, J., Masters, O.E., 3rd, Friedberg, E.C., Lloyd, R.S. and Ward, J.B., Jr. (2006) 3,4-Epoxy-1-butene, a reactive metabolite of 1,3-butadiene, induces somatic mutations in Xpc-null mice. *Environ Mol Mutagen*, **47**, 67-70.

Wilson, T.E. and Lieber, M.R. (1999) Efficient processing of DNA ends during yeast nonhomologous end joining. Evidence for a DNA polymerase beta (Pol4)-dependent pathway. *J Biol Chem*, **274**, 23599-23609.

Winter, C.K., Segall, H.J. and Haddon, W.F. (1986) Formation of cyclic adducts of deoxyguanosine with the aldehydes trans-4-hydroxy-2-hexenal and trans-4-hydroxy-2-nonenal in vitro. *Cancer Res*, **46**, 5682-5686.

Wittschieben, J., Shivji, M.K., Lalani, E., Jacobs, M.A., Marini, F., Gearhart, P.J., Rosewell, I., Stamp, G. and Wood, R.D. (2000) Disruption of the developmentally regulated Rev3l gene causes embryonic lethality. *Curr Biol*, **10**, 1217-1220.

Wittschieben, J.P., Reshmi, S.C., Gollin, S.M. and Wood, R.D. (2006) Loss of DNA polymerase zeta causes chromosomal instability in mammalian cells. *Cancer Res*, **66**, 134-142.

Woodgate, R. (2001) Evolution of the two-step model for UV-mutagenesis. *Mutat Res*, **485**, 83-92.

Yamagami, K., Yamamoto, Y., Kume, M., Ishikawa, Y., Yamaoka, Y., Hiai, H. and Toyokuni, S. (2000) Formation of 8-hydroxy-2'-deoxyguanosine and 4-hydroxy-

2-nonenal-modified proteins in rat liver after ischemia-reperfusion: distinct localization of the two oxidatively modified products. *Antioxid Redox Signal*, **2**, 127-136.

Yamagiwa, K. and Ichikawa, K. (1918) Experimental study of the pathogenesis of carcinoma. *J Cancer Res*, **3**, 1-29.

Yang, I.Y., Chan, G., Miller, H., Huang, Y., Torres, M.C., Johnson, F. and Moriya, M. (2002) Mutagenesis by acrolein-derived propanodeoxyguanosine adducts in human cells. *Biochemistry*, **41**, 13826-13832.

Yang, I.Y., Hossain, M., Miller, H., Khullar, S., Johnson, F., Grollman, A. and Moriya, M. (2001) Responses to the major acrolein-derived deoxyguanosine adduct in Escherichia coli. *J Biol Chem*, **276**, 9071-9076.

Yang, K., Fang, J.L., Li, D., Chung, F.L. and Hemminki, K. (1999) ^{32}P-postlabelling with high-performance liquid chromatography for analysis of abundant DNA adducts in human tissues. *IARC Sci Publ*, 205-217.

Yi, P., Zhan, D., Samokyszyn, V.M., Doerge, D.R. and Fu, P.P. (1997) Synthesis and ^{32}P-postlabeling/high-performance liquid chromatography separation of diastereomeric 1,N^2-(1,3-propano)-2'-deoxyguanosine 3'-phosphate adducts formed from 4-hydroxy-2-nonenal. *Chem Res Toxicol*, **10**, 1259-1265.

Yoritaka, A., Hattori, N., Uchida, K., Tanaka, M., Stadtman, E.R. and Mizuno, Y. (1996) Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease. *Proc Natl Acad Sci U S A*, **93**, 2696-2701.

Yu, S.L., Johnson, R.E., Prakash, S. and Prakash, L. (2001) Requirement of DNA polymerase eta for error-free bypass of UV-induced CC and TC photoproducts. *Mol Cell Biol*, **21**, 185-188.

Zan, H., Komori, A., Li, Z., Cerutti, A., Schaffer, A., Flajnik, M.F., Diaz, M. and Casali, P. (2001) The translesion DNA polymerase zeta plays a major role in Ig and bcl-6 somatic hypermutation. *Immunity*, **14**, 643-653.

Zhang, W., Johnson, F., Grollman, A.P. and Shibutani, S. (1995a) Miscoding by the exocyclic and related DNA adducts 3,N^4-etheno-2'-deoxycytidine, 3,N^4-ethano-2'-deoxycytidine, and 3-(2-hydroxyethyl)-2'-deoxyuridine. *Chem Res Toxicol*, **8**, 157-163.

Zhang, W., Rieger, R., Iden, C. and Johnson, F. (1995b) Synthesis of 3,N^4-etheno, 3,N^4-ethano, and 3-(2-hydroxyethyl) derivatives of 2'-deoxycytidine and their incorporation into oligomeric DNA. *Chem Res Toxicol*, **8**, 148-156.

Zhang, X.Y. and Elfarra, A.A. (2004) Characterization of the reaction products of 2'-deoxyguanosine and 1,2,3,4-diepoxybutane after acid hydrolysis: formation of novel guanine and pyrimidine adducts. *Chem Res Toxicol*, **17**, 521-528.

Zhang, Y., Wu, X., Guo, D., Rechkoblit, O. and Wang, Z. (2002) Activities of human DNA polymerase kappa in response to the major benzo[a]pyrene DNA adduct: error-free lesion bypass and extension synthesis from opposite the lesion. *DNA Repair (Amst)*, **1**, 559-569.

Zhang, Y., Yuan, F., Wu, X., Rechkoblit, O., Taylor, J.S., Geacintov, N.E. and Wang, Z. (2000a) Error-prone lesion bypass by human DNA polymerase eta. *Nucleic Acids Res*, **28**, 4717-4724.

Zhang, Y., Yuan, F., Wu, X., Taylor, J.S. and Wang, Z. (2001) Response of human DNA polymerase iota to DNA lesions. *Nucleic Acids Res*, **29**, 928-935.

Zhang, Y., Yuan, F., Wu, X., Wang, M., Rechkoblit, O., Taylor, J.S., Geacintov, N.E. and Wang, Z. (2000b) Error-free and error-prone lesion bypass by human DNA polymerase kappa in vitro. *Nucleic Acids Res*, **28**, 4138-4146.

Zhang, Y., Yuan, F., Wu, X. and Wang, Z. (2000c) Preferential incorporation of G opposite template T by the low-fidelity human DNA polymerase iota. *Mol Cell Biol*, **20**, 7099-7108.

Zhao, B., Wang, J., Geacintov, N.E. and Wang, Z. (2006) Poleta, Polzeta and Rev1 together are required for G to T transversion mutations induced by the (+)- and (-)-trans-anti-BPDE-N2-dG DNA adducts in yeast cells. *Nucleic Acids Res*, **34**, 417-425.

Zhao, C., Vodicka, P., Sram, R.J. and Hemminki, K. (2000) Human DNA adducts of 1,3-butadiene, an important environmental carcinogen. *Carcinogenesis*, **21**, 107-111.

Zhuang, S.M., Cochran, C., Goodrow, T., Wiseman, R.W. and Soderkvist, P. (1997) Genetic alterations of p53 and ras genes in 1,3-butadiene- and 2',3'-dideoxycytidine-induced lymphomas. *Cancer Res*, **57**, 2710-2714.

Zhuang, S.M., Wiseman, R.W. and Soderkvist, P. (2002) Frequent mutations of the Trp53, Hras1 and beta-catenin (Catnb) genes in 1,3-butadiene-induced mammary adenocarcinomas in B6C3F1 mice. *Oncogene*, **21**, 5643-5648.