THE ACTION OF 1-NITROSO-8-NITROPYRENE IN *ESCHERICHIA COLI*: DNA ADDUCT FORMATION AND MUTATIONAL SPECIFICITY IN THE *LACI* GENE

By

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ABSTRACT

1,8-Dinitropyrene (1,8-DNP) is an environmental contaminant which is mutagenic and carcinogenic. In both prokaryotic and eukaryotic cells 1,8-DNP is metabolized by nitroreduction and O-acetylation to yield a nitrenium ion which binds to DNA. A metabolic intermediate is 1-nitroso-8-nitropyrene (1,8-NONP). This thesis examined the DNA adducts formed by 1,8-NONP, and the mutational specificity of 1,8-NONP in the *lacI* gene of *E. coli*.

Three DNA adducts were formed in *E. coli* following treatment with 1,8-NONP. The major lesion (dG-C(8)-ANP), which comprised about 95% of the total adduct, was formed at the C(8) position of deoxyguanosine. It is likely that one of the minor adducts resulted from reaction with deoxyadenosine, while the other minor adduct was a product of either deoxyguanosine or deoxycytidine.

The mutational specificity of 1,8-NONP was examined by cloning and sequencing several hundred *lacI*$^-$ mutations which had been recovered from four *E. coli* strains which differed with respect to DNA repair background. The results suggested that 1,8-NONP induced many different types of mutations, the most prominent of which were base substitutions, frameshifts and deletions.

Frameshift mutations were induced in all *E. coli* strains tested. However, the extent of frameshift induction was relatively low in *E. coli* strains proficient in nucleotide excision repair, suggesting that the premutagenic adduct(s) can be efficiently recognized and repaired by the *uvrABC* excinuclease. 1,8-NONP-induced

frameshift mutations occurred primarily at sequences of contiguous guanine residues, and were characterized by the loss of G:C base pairs. The frameshift mutations exhibited marked site specificity, consistent with an "incorporation-slippage" model for frameshift mutation. In some cases inverted and direct repeats might contribute to frameshift mutagenesis. The vast majority of frameshift mutations occurred at G:C base pairs, and were probably targeted by the major dG-C(8)-ANP adduct.

Base substitution mutation was strongly influenced by cellular error-prone repair functions. Most of the base substitutions were G:C => T:A transversions and were probably targeted by the major dG-C(8)-ANP adduct. However, a small number of A:T => T:A transversions might have been targeted by the minor deoxyadenosine adduct. The nature of the base substitutions induced by 1,8-NONP suggests that adenine is the most common base incorporated during error-prone bypass of the bulky DNA adducts.

The endpoints of most deletion mutations were G:C rich and contained direct repeats, consistent with a slippage model for deletion mutation. Some deletion endpoints contained sequences which were similar to the putative recognition sequences for enzymes which nick DNA during replication and repair.

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LIST OF ABBREVIATIONS

µl	microlitre
µmol	micromole
1-HAP	1-N-hydroxylaminopyrene
1-NOP	1-nitrosopyrene
1-NP	1-nitropyrene
1,3-DNP	1,3-dinitropyrene
1,3,6-TNP	1,3,6-trinitropyrene
1,3,6,8-TNP	1,3,6,8-tetranitropyrene
1,6-ANP	1-amino-6-nitropyrene
1,6-DNP	1,6-dinitropyrene
1,6-NONP	1-nitroso-6-nitropyrene
1,8-AANP	N-acetoxy-1-amino-8-nitropyrene
1,8-ANP	1-amino-8-nitropyrene
1,8-DAP	1,8-diaminopyrene
1,8-DNP	1,8-dinitropyrene
1,8-HANP	N-hydroxy-1-amino-8-nitropyrene
1,8-NONP	1-nitroso-8-nitropyrene
2-NP	2-nitropyrene
2,7-DNP	2,7-dinitropyrene
A	adenine
AAF	2-acetylaminofluorene
AF	2-aminofluorene
AF2	2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide
AFB_1	aflatoxin B_1

AFC^R	resistance to 1-β-arabinofuranosyl cytosine.
AG sites	aguaninic sites
Amp	Ampicillin
AP sites	apurinic sites
AT	acetyltransferase
ATP	adenosine 5'-triphosphate
BP	Benzo(a)pyrene
BPDE	Benzo(a)pyrenediolepoxide
C	cytosine
CHL	Chinese hamster lung
CHO	Chinese hamster ovary
CHP	Chinese hamster pulmonary cells
CHV79	Chinese hamster lung V79
Ci	Curie
CT DNA	Calf thymus DNA
dA	deoxyadenosine
dC	deoxycytidine
ddNTP	dideoxynucleoside triphosphates
dG	deoxyguanosine
dG-C(8)-ANP	N-(2'-deoxyguanosin-8-yl)-1-amino-8-nitropyrene
dG-C(8)-AP	N-(2'-deoxyguanosin-8-yl)-1-aminopyrene
DHF	diploid human fibroblasts
DHF-XP	diploid human fibroblasts obtained from a patient with xeroderma pigmentosum (XP)
Dip. $toxin^R$	resistance to diptheria toxin
DMF	dimethylformamide

dNTP	deoxynucleoside triphosphates
dT	deoxythymidine
E. coli	Escherichia coli
F344	Fisher 344
G	guanine
Glu	Glucose
HGPRT	hypoxanthine guanine phosphoribosyl transferase
Human hep.	human hepatocytes
i.p.	intraperitoneal injection
i.t.i.	intratracheal instillation
LB	Luria-Bertani
Met^R	resistance to methotrexate
MFH	malignant fibrous histiocytomas
ML L5178Y	Mouse lymphoma L5178Y
ml	millilitre
mmol	millimole
mouse hep.	mouse hepatocytes
NAAP	N-acetylamino-hydroxypyrenes
Nal	Naladixic Acid
ND	not determined
NGGN	2 contiguous guanine residues flanked by A, T, or C on the 3'- and 5'-ends
AGGN	2 contiguous guanine residues flanked by A on the 5'- end and A, T, or C on the 3'-end
NGGA	2 contiguous guanine residues flanked by A, T, or C on the 5'-end, and A on the 3'- end
nmol	nanomole

NR	nitroreductase
Oua^R	resistance to ouabain
PAHs	polycyclic aromatic hydrocarbons
paint	skin painting
PEG	polyethylene glycol
PEI	polyethyleneimine
PGal	phenyl-β-D-galactoside
PNK	T4 polynucleotide kinase
PolI	DNA polymerase I
PolII	DNA polymerase II
PolIII	DNA polymerase III
ppm	parts per million
Rat hep.	rat hepatocytes
RERL4	Rat epithelium cell line RL4
Rif	Rifampicin
S. typhimurium	Salmonellla typhimurium
s.c.	subcutaneous injection
SCEs	sister chromatid exchanges
SDS	sodium dodecyl sulfate
SFS	synchronous fluorescence spectrophotometry
SOI	site of inection
Strp	Streptomycin
T	thymine
TBAC	tetrabutylammonium chloride
TG^R	resistance to thioguanine
TLC	thin layer chromatography

TONPG	O-nitrophenyl-β-D-thiogalactoside
UDS	unscheduled DNA synthesis
VB	Vogel-Bonner
XGal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Glossary

Although the term *frameshift* applies to any gain or loss of bases which results in a change of the reading frame during translation, its use in this thesis is restricted to mutations involving the gain or loss of one or two bases. Loss of three or more bases will be referred to as *deletions*; reiteration of 3 or more bases will be referred to as *duplications*; and nonduplicative insertion of 3 or more bases will be referred to as *additions*.

The *uvrABC excinuclease* refers to the complex formed by the products of the *uvrA*, *uvrB*, and *uvrC* genes. The combined activity of this complex, DNA helicase II and DNA polymerase I result in the recognition and excision of several forms of DNA damage.

1. INTRODUCTION

Many chemicals which are present in the environment are capable of interacting with DNA to induce mutations in both prokaryotic and eukaryotic cells. A large number of these chemicals are inactive *per se* and require biotransformation to a species which can react with DNA. The formation of covalent bonds between the active form of the mutagen and sites within the DNA duplex yields DNA adducts which are important premutagenic lesions. The biological consequences of DNA adduction is determined by the nature of the lesion, and the activity of several cellular factors. DNA repair enzymes often recognize and remove the adducts by error-free mechanisms; this constitutes an efficient strategy by which cells can tolerate DNA damage. However, when DNA which contains adducts is replicated prior to repair, replicational errors frequently occur.

DNA adducts fall into two general classes which are believed to give rise to mutations by different mechanisms. Members of one class are mutagenic as a consequence of their ability to mispair during normal DNA replication (Drake and Baltz, 1976). DNA lesions of the second class are replicated only with difficulty; in bacteria, and perhaps also in mammalian cells, the ability of such lesions to cause mutations is at least partially dependent on cellular functions which are intrinsically error-prone (Walker, 1984).

Several chemical mutagens also induce tumors in mammals. Carcinogenesis is a multistage process involving the sequential

evolution of genetically altered cell populations (Nowell, 1986; Klein, 1987). A number of different types of genes, including proto-oncogenes (Bishop, 1987), tumor suppressor genes (Knudsen, 1985; Klein, 1987), and genes which modulate functions such as DNA repair, mitosis, DNA replication, tumor invasiveness, and metastasis (Nowell, 1986; Klein, 1987), are known to influence the growth of normal and tumor cells. It has been postulated that mutagens contribute to tumorigenesis by inducing genomic alterations which change the expression or biochemical function of these genes (Nowell, 1986; Weinstein, 1988).

The combustion of organic fuels is an important source of environmental mutagens and carcinogens. The combustion products which have been most extensively investigated are the polycyclic aromatic hydrocarbons (PAHs). The chemistry, metabolism, carcinogenicity, and mutagenicity of PAHs has been documented extensively in the scientific literature for more than 50 years. A related class of compounds, the nitroarenes, have only recently been identified as potentially hazardous environmental contaminants. In 1979, Pitts and coworkers demonstrated that exposure of the PAHs benzo(a)pyrene or perylene to atmospheres containing ppm quantities of NO_2 and traces of nitric acid gave rise to nitrated products which were direct acting mutagens in the Salmonella reversion assay (Pitts et al., 1979). These authors noted that relatively high levels of both PAH and oxides of nitrogen were common conditions in a wide range of combustion processes, as well as in the atmosphere of industrialized areas, and suggested that the nitroarene products

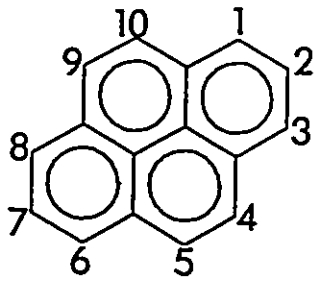
might be of environmental importance. Since that time a large number of nitroarenes exhibiting a wide variety of biological activities have been identified (Schuetzle et al., 1982; Rosenkranz and Mermelstein, 1983; Tokiwa and Ohnishi, 1986).

Among the nitroarenes, the nitropyrenes (Figure 1) are particularly prominent. These compounds are nitrated derivatives of pyrene (a four ring PAH) which have been identified in jet and diesel exhaust emissions (Scheutzle et al., 1982; Rosenkranz, 1982; Rosenkranz and Mermelstein, 1983; McCartney et al., 1986), the carbon blacks of xerographic toners used prior to 1979 (Rosenkranz and Mermelstein, 1980), cigarette smoke (McCoy and Rosenkranz, 1982; El-Bayoumy et al., 1985), urban air particulates (Tokiwa et al., 1983; Rosenkranz and Mermelstein, 1983; Tokiwa and Ohnishi, 1986; Arey et al., 1988), and grilled chicken (Kinouchi et al., 1986). These compounds are potent mutagens in bacteria, are both mutagenic and clastogenic in mammalian cells in culture, and are carcinogenic in rodents (reviewed in Rosenkranz et al., 1980; Rosenkranz and Mermelstein, 1983; Tokiwa and Ohnishi, 1986).

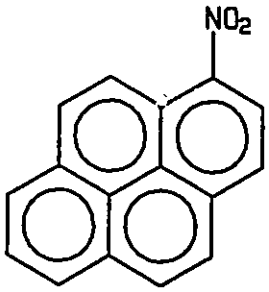
1.1 Genetic Toxicology of Nitropyrenes

1.1.1 Mutagenicity in Bacteria

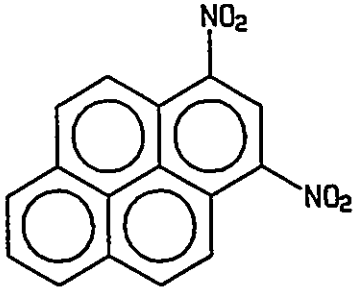
Nitropyrenes exhibit strong mutagenic activity in the Salmonella typhimurium tester strains developed by Ames and coworkers (Maron and Ames, 1983; Hartman et al., 1986). The mutagenicity is direct acting (i.e. does not require the presence of rat liver S-9 extracts) and is, in fact, substantially reduced by the addition of S9 activation mixtures to the treatment medium (Rosenkranz et al.,



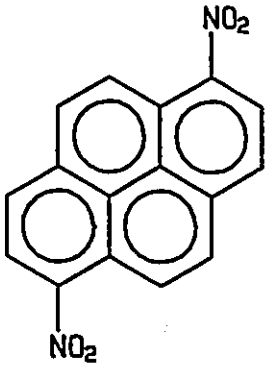
Pyrene



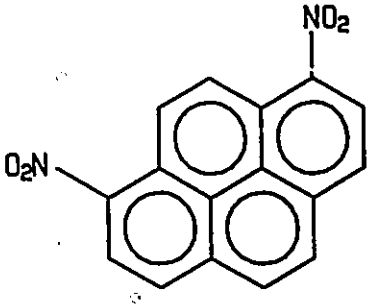
1-nitropyrene (1-NP)



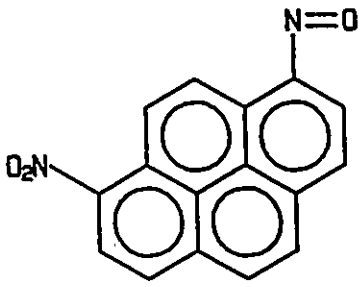
1,3-dinitropyrene (1,3-DNP)



1,6-dinitropyrene (1,6-DNP)



1,8-dinitropyrene (1,8-DNP)



1-nitroso-8-nitropyrene (1,8-NONP)

Figure 1. Structures of several nitropyrene derivatives.

1980; Mermelstein et al., 1981). Table 1 shows the mutagenicity of nitropyrene derivatives in several tester strains. Nitropyrenes induce both frameshift and base substitution mutations as reflected by the activity of several of these compounds in strains TA98 and TA100, respectively. Dinitropyrenes are the most potent mutagens of this class, followed by the trinitro-, tetranitro- and mononitro- derivatives, in that order. The difference in mutagenic potency between the nitropyrenes is marked: mutation rates in TA98 range from about 250000 revertants per nanomole for 1,8-dinitropyrene (1,8-DNP) to less than 500 revertants per nanomole for 1-nitropyrene (1-NP) (Rosenkranz, 1982; Rosenkranz and Mermelstein, 1983, Tokiwa and Ohnishi, 1986, Hirayama et al., 1988). The former value places 1,8-dinitropyrene among the most potent bacterial mutagens tested to date (Rosenkranz and Mermelstein, 1983).

The mutagenic potency of nitropyrenes is increased in bacterial strains containing the plasmid pKM101, which encodes cellular error-prone repair functions. This is particularly the case at the base substitution locus hisG46 (strains TA1535 and TA100), where no mutagenic activity is detected in the absence of pKM101 (Table 1). At the frameshift locus hisD3052 (TA1978, TA1538 and TA98), the mutation frequency is increased 2 to 10-fold in the strain containing plasmid pKM101 (TA98) relative to the otherwise isogenic strain which lacks the plasmid (TA1538). Mutagenic activity is greatly reduced in strains with functional nucleotide excision repair activity. This suggests that the premutagenic lesion is one which is recognized by the S. typhimurium uvrABC exinuclease and efficiently

Table 1. The mutagenicity of nitropyrenes in S. typhimurium tester strains. The values given in this table are revertants per nanomole.

[a] The histidine mutations in the S. typhimurium tester strains have been described recently (Maron and Ames, 1983; Hartman et al., 1986). Tester strains containing the hisG46 and hisG428 loci are believed to revert as a result of base substitution mutations, while strains containing the hisC3076, hisD3052, and hisD6580 loci revert primarily as a consequence of frameshift mutations. Mutations which revert the hisG46, hisC3076, and hisD3052 loci occur most frequently at G:C base pairs, while those at the hisG428 and hisD6580 loci occur primarily at A:T base pairs (reviewed in Hartman et al., 1986).

[b] Nucleotide excision repair: +, strains which are proficient in nucleotide excision repair; -, strains which are deficient in nucleotide excision repair capability due to a deletion through the uvrB locus.

[c] Plasmid pKM101: +, strains containing the plasmid pKM101 which enhances error-prone repair; -, strains which do not contain pKM101.

[d] Abbreviations: 1-NP, 1-nitropyrene; 2-NP, 2-nitropyrene; 1,3-DNP, 1,3-dinitropyrene; 1,6-DNP, 1,6-dinitropyrene; 1,8-DNP, 1,8-dinitropyrene; 2,7-DNP, 2,7-dinitropyrene; 1,3,6-TNP, 1,3,6-trinitropyrene; 1,3,6,8-TNP, 1,3,6,8-tetranitropyrene.

[e] ND, not determined.

Table 1. The mutagenicity of nitropyrenes in S. typhimurium tester strains.

histidine mutation [a]	hisG46		hisC3076		his D3052			hisG428	hisD6580	
strain	TA1535	TA100	TA1977	TA1537	TA1978	TA1538	TA98	TA102	TA96	Reference
uvrABC repair[b]	-	-	+	-	+	-	-	+	-	
pKM101[c]	-	+	-	-	-	-	+	+	+	
1-NP[d]	0	119	ND	34	ND	120	46	ND	ND	Tokiwa & Ohnishi, 1986
	ND[e]	ND	ND	ND	ND	ND	673	ND	ND	Hirayami et al., 1988
	ND	84	ND	ND	ND	ND	453	9	321	Massaro et al., 1983
	0	63	0	67	0	85	484	ND	ND	Mermelstein et al., 1981
2-NP	ND	ND	ND	ND	ND	ND	2568	ND	ND	Hirayama et al., 1988
1,3-DNP	0	19204	ND	8220	ND	13412	130215	ND	ND	Tokiwa & Ohnishi, 1986
	ND	ND	ND	ND	ND	ND	99659	ND	ND	Hirayama et al., 1988
	ND	10880	ND	ND	ND	ND	145065	190	8054	Massaro et al., 1983
		8359	0	13400	0	15600	28600	ND	ND	Mermelstein et al., 1981
1,6-DNP	0	21616	ND	22312	ND	11928	174842	ND	ND	Tokiwa & Ohnishi, 1986
	0	5427	ND	ND	ND	ND	183960	2068	39817	Massaro et al., 1983
	0	ND	303	33000	0	12100	36350	ND	ND	Mermelstein et al., 1981
1,8-DNP	0	55420	ND	16189	ND	19532	257439	ND	ND	Tokiwa & Ohnishi, 1986
	ND	5548	ND	ND	ND	ND	254040	262	29328	Massaro et al., 1983
	0	ND	0	11800	ND	9950	72900	ND	ND	Mermelstein et al., 1981
2,7-DNP	ND	ND	ND	ND	ND	ND	37960	ND	ND	Hirayama et al., 1988
1,3,6-TNP	0	17383	ND	23735	ND	25181	36985	ND	ND	Tokiwa and Ohnishi, 1986
	0	5750	37	20100	7	15650	31400	ND	ND	Mermelstein et al., 1981
	ND	1256	ND	ND	ND	ND	20489	285	32048	Massaro et al., 1983
1,3,6,8-TNP	0	3580	ND	3157	ND	1884	15211	ND	ND	Tokiwa & Ohnishi, 1986
	0	750	6	3300	3	1350	7700	ND	ND	Mermelstein et al., 1981
	ND	131	ND	ND	ND	ND	4133	6	3954	Massaro et al., 1983

see footnote on opposite page

repaired. Mutagenicity is observed in strains containing either G:C (hisG46, hisC3076, and hisD3052) or A:T (hisD6580 and hisG428) base-pairs at the putative mutational hotspot.

Initially, nitropyrenes were reported to be nonmutagenic in Escherichia coli (Mermelstein et al., 1981). However, subsequent studies demonstrated that this resistance was due to a permeability barrier to nitropyrenes which is not present in most of the S. typhimurium tester strains (which are deficient in the polysaccharide component of the lipopolysaccharide coat and thus allow the entry of a number of large chemicals) (McCoy et al., 1985a). Permeability mutants of E. coli strain WP2uvrA were constructed and shown to be sensitive to 1-NP, 1,3-dinitropyrene (1,3-DNP), 1,6-dinitropyrene (1,6-DNP), and 1,8-DNP; induced base substitution mutations were observed only in those E. coli strains which contained the plasmid pKM101 (McCoy et al., 1985a).

The precise nature of the mutational changes induced by nitropyrenes is unknown. Although Ames tester strains frequently revert by specific base changes at a "mutational hotspot", the results are somewhat ambiguous: recent studies suggest that reversion of several loci can occur as the result of mutational events at several sites within the histidine genes, and even by extragenic suppressor mutations (reviewed by Hartman et al., 1986). Therefore without further characterization of the revertants, the type and site of mutation cannot be known with certainty. Only one study to date has used DNA sequencing to examine the base changes in forward mutations induced in bacteria by nitropyrene derivatives.

The DNA sequence of 30 lambda phage cI mutations was determined following treatment of the E. coli host with 1-nitrosopyrene (1-NOP), a partially reduced (metabolically activated) form of 1-NP. The majority of the 1-NOP-induced mutations were single base frameshifts at G:C base-pairs (Stanton et al., 1988). However, these workers were unable to evaluate the mutational specificity in depth due to the relatively small number of mutations in the collection.

1.1.2 Mutagenicity and Clastogenicity in Cultured Eukaryotic Cells

Studies using cultured eukaryotic cells suggest that nitropyrenes are also genotoxic in higher organisms (Table 2). Early studies demonstrated that 1,8-DNP was mutagenic in mouse lymphoma L5178Y cells using resistance to 6-thioguanine, methotrexate, ouabain, or 1-β-arabinofuranosyl cytosine as selective markers (Cole et al., 1982). 1,8-DNP, 1,6-DNP and 1,3,6-TNP were direct acting mutagens at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus of Chinese hamster ovary (CHO) cells; the induced mutation frequency was decreased by the addition of Aroclor-induced S9 to the treatment medium (Li and Dutcher, 1983). In contrast, 1-NP was only slightly mutagenic at the HGPRT locus of CHO or Chinese hamster lung V79 (CHV79) cells, and the mutation frequency was increased approximately 4-fold in both cell types by the addition of S9 to the treatment medium (Li and Dutcher, 1983; Berry et al., 1985). These data suggest that oxidative metabolism can play a role in activating 1-NP to a species which reacts with DNA (Li and Dutcher, 1983; Berry et al., 1985). In Chinese hamster lung (CHL)

Table 2. Mutagenicity and clastogenicity of nitropyrenes in cultured mammalian cells.

[a] Abbreviations for cell types: CHL, Chinese hamster lung; CHO, Chinese hamster ovary; CHV79, Chinese hamster lung V79; DHF, diploid human fibroblasts; DHF-XP; diploid human fibroblasts obtained from a patient with xeroderma pigmentosum (XP); Rat hep., rat hepatocytes; mouse hep., mouse hepatocytes; Human hep., human hepatocytes; CHP, Chinese hamster pulmonary cells; RERL4, Rat epithelium cell line RL4; ML L5178Y, Mouse lymphoma L5178Y.

[b] Abbreviations for genetic marker: Dip. toxin^R, resistance to diptheris toxin; HGPRT, hypoxanthine-guanine phosphoribosyl transferase; TG^R, resistance to thioguanine; SCEs, sister chromatid exchanges; UDS, unscheduled DNA synthesis; Met^R, resistance to methotrexate; Oua^R, resistance to ouabain; AFC^R, resistance to 1-ß-arabinofuranosyl cytosine.

Table 2. Mutagenicity and clastogenicity of nitropyrenes in cultured mammalian cells.

	Dose (μM)	Mutagenicity or Clastogenicity	Cells	Genetic Marker & Units	Reference
1-NP	1	<1	CHL	Dip.toxin^R mutants/10^6 sur.	Nakayasu et al., 1982
	120	7.2	CHO	HGPRT mutants/10^6 survivors	Li & Dutcher, 1983
	40	0	CHV79	Ouabain^R mutants/10^6 survovors	Takayama et al., 1983
	50	18 (-S9)	CHV79	HGPRT mutants/10^6 survivors	Berry et al., 1985
	50	72 (+S9)	CHV79	HGPRT mutants/10^6 survivors	Berry et al., 1985
	60	101	DHF	TG^R mutants/ 10^6 survivors	Patton et al., 1986
	13.8	162	DHF-XP	TG^R mutants / 10^6 survivors	Patton et al., 1986
	30	13	CHO	SCEs/cell	Nachtman & Wolff, 1982
	142	8.7	Rat Hep.	UDS grains/ nucleus	Mori et al., 1987
	142	8.6	Mouse Hep.	UDS grains/ nucleus	Mori et al., 1987
	120	10%	CHP	Chromatid aberrations (% cells)	Lafi & Parry, 1987
1,3-DNP	1	74	CHL	Dip.toxin^R mutants/10^6 sur.	Nakayasu et al., 1982
	3.5	5.1	CHO	HGPRT mutants/10^6 survivors	Li & Dutcher, 1983
	34	9.2	CHV79	Ouabain^R mutants/10^6 survivors	Takayama et al., 1983
	38	114	Rat Hep.	UDS grains/ nucleus	Mori et al., 1987
	38	98	Mouse Hep.	UDS grains/ nucleus	Mori et al., 1987
1,6-DNP	1	210	CHL	Dip.toxin^R mutants/ 10^6 sur.	Nakayasu et al., 1982
	7	17	CHO	HGPRT mutants/ 10^6 survivors	Li & Dutcher, 1983
	4	64%	RERLA	Chromatid aberrations (% cells)	Danford et al., 1982
	4	10%	RERLA	Chromosomal aberrations (%cells)	Danford et al., 1982
	5	54%	CHV79	Chromatid aberrations (% cells)	Bauchinger et al., 1988
	5	37	Human Hep.	UDS grains/ nucleus	Butterworth et al., 1983
	34	88	Rat. Hep.	UDS grains/ nucleus	Mori et al., 1987
	34	51	Mouse Hep.	UDS grains/ nucleus	Mori et al., 1987
1,8-DNP	4.2	10 to 100	ML L5178Y	TG^R, Mat^R, Oua^R, AFC^R mutants per 10^6 survivors	Cole et al., 1982
	1	152	CHL	Dip.toxin^R mutants/ 10^6 sur.	Nakayasu et al., 1982
	7	49	CHO	HPRT^R mutants/ 10^6 survivors	Li & Dutcher, 1983
	0.3	39.4	CHV79	Ouabain^R mutants/ 10^6 survivors	Takayama et al., 1983
	4	60%	RERLA	Chromatid aberrations (% cells)	Danford et al., 1982
	4	18%	RERLA	Chromosomal aberrations (%cells)	Danford et al., 1982
	6	13.7	CHO	SCEs/ cell	Nachtmann & Wolff, 1982
	34	102	Rat Hep.	UDS grains/ nucleus	Mori et al, 1987
	34	70	Mouse Hep.	UDS grains/ nucleus	Mori et al., 1987
1,3,6-TNP	1	65	CHL	Dip.toxin^R mutants/ 10^6 sur.	Nakayasu et al., 1982
	6	120	CHO	HGPRT mutants/ 10^6 survivors	Li & Dutcher, 1983
1,3,6,8-TNP	1	<1.5	CHL	Dip. toxin^R mutants/ 10^6 sur.	Nakayasu et al., 1982

see footnotes on opposite page

cells treatment with 1-NP at a dose of 1 μM failed to induce resistance to diptheria toxin. However, a similar dose of 1,8-DNP, 1,6-DNP, 1,3-DNP or 1,3,6-TNP was highly mutagenic in CHL cells (Nakayasu et al., 1982). Only one laboratory has examined nitropyrene-induced mutagenicity in human cells; Patton et al. (1986) showed that 1-NP was mutagenic in diploid human fibroblasts derived from either xeroderma pigmentosum (XP) or normal individuals. As shown in Table 2, the XP cells, which are deficient in nucleotide excision repair capability, were considerably more sensitive to 1-NP than the normal cells.

Both 1-NP and 1,8-DNP have been shown to cause a dose dependent increase in sister chromatid exchanges (SCEs) in CHO cells, with 1,8-DNP being active at a much lower dose (Nachtman and Wolff, 1982). Several studies have examined nitropyrene-treated cells for chromosomal and chromatid aberrations: 1,8-DNP and 1,6-DNP were potent clastogens in RL_4 rat epithelial cells and CHV79 cells (Danford et al., 1982; Baushinger et al., 1988), while 1-NP elicited a weak, but statistically significant, clastogenic effect in Chinese hamster pulmonary cells (Lafi and Parry, 1987). Dose related increases in unscheduled DNA synthesis were induced by 1,6-DNP in primary cultures of human hepatocytes (Butterworth et al., 1983), and by 1-NP, 1,3-DNP, 1,6-DNP and 1,8-DNP in rat and mouse hepatocytes (Mori et al., 1987). In this test the dinitropyrene congeners exhibited considerably higher genotoxicity than 1-NP.

Maher and colleagues have examined the types of mutations induced by 1-NOP in mammalian cells (Maher et al., 1987; Yang et al.,

1989). 1-NOP was reacted with the shuttle vector pZ189, and the plasmid was then introduced into human cells; G:C => T:A transversions were the most common mutation recovered in the supF gene of pZ189 (Yang et al., 1989). The same workers demonstrated that treatment of mouse L cells with 1-NOP induces homologous recombination between duplicated DNA sequences (Maher et al., 1987).

1.1.3 Carcinogenicity

Following the demonstration that nitropyrenes were potent mutagens, several studies examined the carcinogenicity of these compounds (Table 3). The first report of tumor induction by nitropyrenes in experimental animals showed that sarcomas were induced at the site of subcutaneous (s.c.) injection by 1-NP in Fisher 344 (F344) rats (Ohgaki et al., 1982). It was later found that the 1-NP used had contained traces of dinitropyrene congeners. Repetition of the experiment with highly purified 1-NP did not produce any site of injection tumours by 650 days when the experiment was terminated. However, rats treated with 1,6-DNP or 1,8-DNP all developed tumours within 320 days (Ohgaki et al., 1985). In a separate study, the same investigators showed that s.c. injection of 1,3-DNP produced tumours at the site of injection in all of the rats tested (Ohgaki et al., 1984). These results are consistent with the findings of Tokiwa et al. (1984) who demonstrated that 1,6-DNP produced site of injection tumors following s.c. injection in BALB/c mice. Recently King (1988) has demonstrated that the nitropyrenes exhibit different tumorigenicity following s.c. injection in newborn CD rats: treatment with 6.3 μmol of 1-NP or 1,3-DNP caused only a

Table 3. Carcinogenicity of nitropyrenes.

	Dose	Route[a]	Animal[b]	Induced tumors[c]	Reference
1-NP	800 µmole/kg	s.c.	CD rat (M&F)	MFH (SOI) (M:32%; F:28%) Mammary (F:47%)	Hirose et al., 1984
	6400 µmole/kg	i.p.	A/J mice (M&F)	Lung (M:88%; F:67%)	El-Bayoumy et al., 1984
	162 µmole	s.c.	F344 rat (M)	None	Ohgaki et al., 1985
	2.8 µmole	i.p.	Newborn CD-1 mice (M&F)	Liver (M:28%)	Wislocki et al., 1986
	4 µmole	paint	Crl/CD-1 BR mice	None	El-Bayoumy et al., 1984
	800 µmole	gavage	SpragueDawley rats (M&F)	Mammary adenocarcinoma (F:63%)	El-Bayoumy et al., 1988
	16 µmole	i.p.	CD rats (F)	Mammary (69%)	King, 1988
	16 µmole	gavage	CD rats (F)	None	King, 1988
	6.3 µmole	s.c.	Newborn CD rat (F)	Mammary (33%)	King, 1988
	40 µmole	s.c.	Newborn F344 rat (F)	None	King, 1988
1,3-DNP	14 µmole	s.c.	F344/DuCrj rat (M)	Fibrosarcoma (SOI) (100%)	Ohgaki et al., 1984
	0.2 µmole	i.p.	Newborn CD-1 mice (M&F)	Liver (M:20%)	Wislocki et al., 1986
	16 µmole	i.p.	CD rats (F)	None	King, 1988
	16 µmole	gavage	CD rats (F)	None	King, 1988
	6.3 µmole	s.c.	Newborn CD rats (F)	MFH (SOI) (12%)	King, 1988
1,6-DNP	14 µmole	s.c.	F344/DuCrj rat (M)	Fibrosarcoma (SOI) (100%)	Ohgaki et al., 1985
	7 µmole	s.c.	BALB/C mice (M)	MFH (SOI) (50%)	Tokiwa et al., 1984
	44 µmole	i.t.i.	Syrian Golden Hamster (M&F)	Lung (M:100%; F:90%) Leukemia (M:60%; F:60%)	Takayama et al., 1985
	0.2 µmole	i.p.	Newborn CD-1 mice (M&F)	Liver (M:32%)	Wislocki et al., 1986
	16 µmole	i.p.	CD rats (F)	MFH (SOI) (100%)	King, 1988
	16 µmole	gavage	CD rats (F)	none	King, 1988
	6.3 µmole	s.c.	Newborn CD Rats	MFH (SOI) (100%), Leukemia (20%)	King, 1988
1,8-DNP	0.14 µmole	s.c.	F344/DuCrj rat (M)	Fibrosarcoma (SOI) (90%)	Ohgaki et al., 1985
	1.4 µmole	s.c.	F344/DuCrj rat (M)	Fibrosarcoma (SOI) (100%)	Ohgaki et al., 1985
	0.2 µmole	i.p.	Newborn CD-1 mice (M&F)	Liver (M:16%)	Wislocki et al., 1986
	16 µmole	i.p.	CD rats (F)	MFH (SOI) (88%) Mammary adenocarcinoma (42%) Leukemia (21%)	King, 1988
	16 µmole	gavage	CD rats (F)	None	King, 1988
	6.3 µmole	s.c.	Newborn CD rats (F)	MFH (SOI) (100%), Leukemia (22%)	King, 1988

[a] Abbreviations for route of exposure: s.c., subcutaneous injection; i.p., intraperitoneal injection; paint, skin painting; i.t.i., intratracheal instillation.
[b] F, female; M, male; M&F, carcinogenicity was examined in both males and females.
[c] Abbreviations for tumor type: MFH, malignant fibrous histiocytomas; SOI, site of inection.

slight induction in mammary and site of injection tumors, respectively; however, all animals treated with a similar dose of 1,6-DNP or 1,8-DNP developed site of injection tumors, and about 20 % of the animals developed leukemia. The mean survival of the animals treated with 1,6-DNP or 1,8-DNP was about one third that of the rats treated with equimolar doses of 1-NP or 1,3-DNP (King, 1988).

Nitropyrenes are also carcinogenic when administered by intraperitoneal (i.p.) injection (Table 3). Different studies have shown that i.p. injection with 1-NP results in the induction of lung tumors in A/J mice (El-Bayoumy et al., 1984a), liver tumors in CD-1 mice (Wislowski et al., 1986), and mammary tumors in CD rats (King, 1988). Intraperitoneal injection of newborn rodents with 1,8-DNP or 1,6-DNP resulted in the induction of liver tumors (Wislowski et al., 1986), mammary tumors, and leukemias (King, 1988). However, the most prominent tumors induced by i.p. injection of 1,8-DNP or 1,6-DNP were malignant fibrous histiocytomas (MFHs) at the site of injection (King, 1988).

Intratracheal instillation of 1,6-DNP produced lung carcinomas in both male and female Syrian golden hamsters. In addition, 60 % the animals of each sex treated with 1,6-DNP by intratracheal installation developed myeloid leukemias (Takayama et al., 1985). This study is particularly pertinent since the most common route of human exposure to nitropyrenes is likely to be via inhalation.

The only studies to date which have examined genetic changes in nitropyrene-induced tumors have characterized alterations in the

oncogene K-ras in fibrosarcomas induced by s.c. injection of 1,8-DNP (Ochai et al., 1985). The activated K-ras contained a G:C => T:A transversion in codon 12 (Tahira et al., 1986).

1.2 Metabolism of Nitropyrenes and Formation of DNA Adducts

1.2.1 Bacterial Metabolism

Bacteria contain reductases which are capable of reducing the nitro moiety of many nitroheterocyclic and nitroaromatic compounds (Peterson et al., 1979; McCoy et al., 1981; McCalla, 1983; Bryant et al., 1984). The combined activity of the nitroreductases, and bacterial acetyltransferases (McCoy et al., 1983; Orr et al., 1985; Saito et al., 1985) metabolize nitropyrenes to relatively innocuous endproducts. Thus, in S. typhimurium, 1-NP is metabolized to 1-aminopyrene and N-acetyl-1-aminopyrene (Messier et al., 1981). Similarly, end products of 1,8-DNP metabolism include 1-amino-8-nitropyrene, 1,8-diaminopyrene, 1-acetylamino-8-nitropyrene, 1-acetylamino-8-aminopyrene, and 1,8-diacetylaminopyrene (Figure 2) (Bryant et al., 1984; Orr et al., 1985). In contrast to the metabolic endproducts, the hydroxylamine intermediates formed during reductive metabolism of nitro groups are extremely unstable, and are believed to be the proximate mutagens. Hydroxylamines can be converted either enzymatically or nonenzymatically to a reactive nitrenium ion which can bind to macromolecules (Rosenkranz and Mermelstein, 1983; McCoy et al., 1983; Kadlubar and Beland, 1985; Tokiwa and Ohnishi, 1986; King, 1988).

A great deal of useful information regarding nitropyrenes has been obtained from studies using S. typhimurium strains TA98NR and

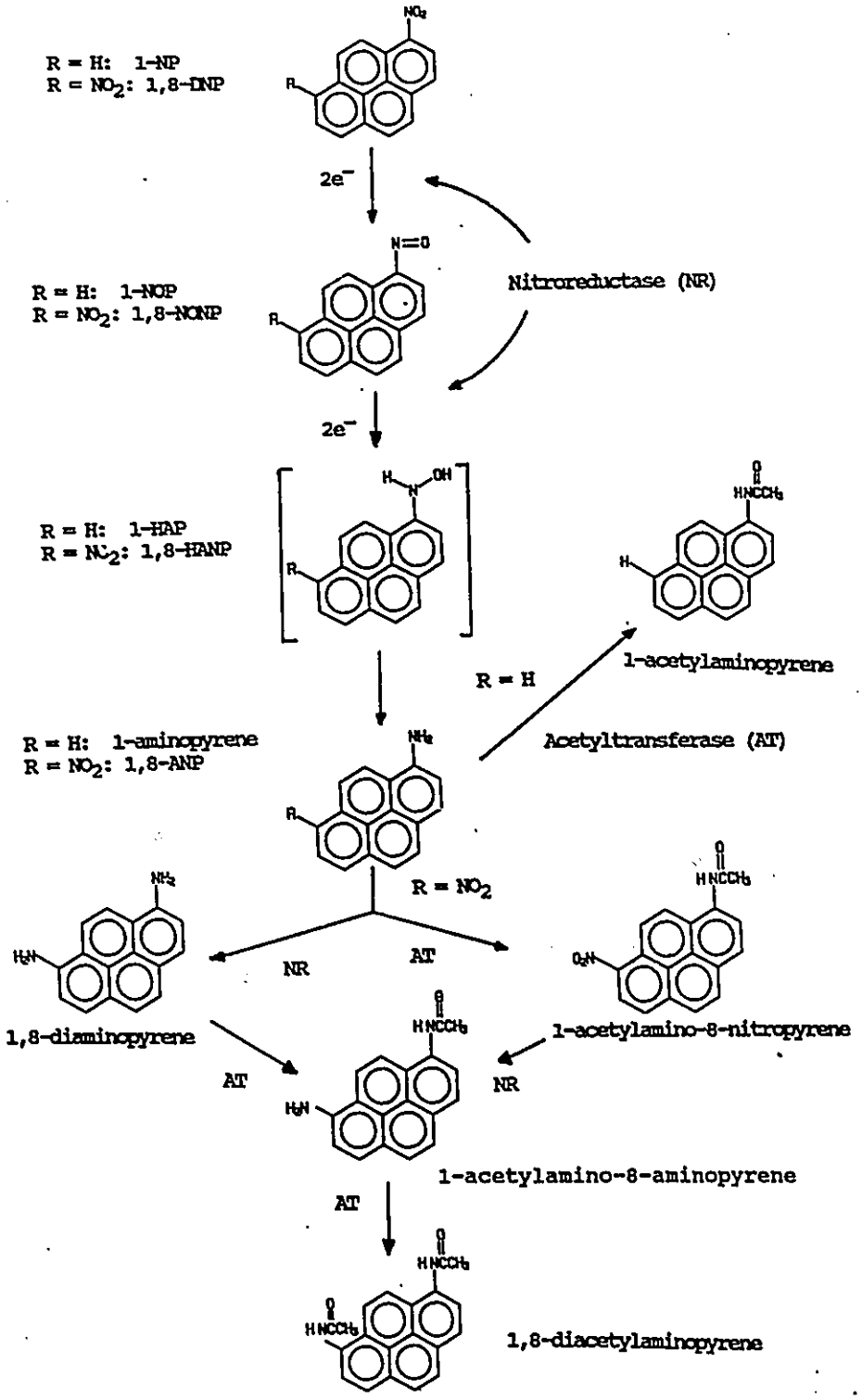


Figure 2. Metabolism of 1-NP and 1,8-DNP in bacteria.
[a] Abbreviations: 1-NP, 1-nitropyrene; 1-NOP, 1-nitrosopyrene; 1-HAP, N-hydroxy-1-aminopyrene; 1,8-DNP, 1,8-dinitropyrene; 1,8-NONP, 1-nitroso-8-nitropyrene; 1,8-HANP, N-hydroxy-1-amino-8-nitropyrene; 1,8-ANP, 1-amino-8-nitropyrene; AT, acetyltransferase; NR, nitroreductase.

TA98/1,8-DNP$_6$. The mutagenicity of nitropyrenes and nitropyrene metabolites in these strains is shown in Table 4. The mutagenic activity of 1-NP and 1,3-DNP decreases markedly in strain TA98NR; this correlates with a diminution in the nitroreductase activity observed in cell free extracts of TA98NR (Bryant et al., 1984). The mutagenicity of the partially reduced 1-nitrosopyrene derivative is the same in strains TA98 and TA98NR; this provides additional evidence that TA98NR is defective in nitroreductase activity. 1,6-DNP and 1,8-DNP are equally mutagenic in both TA98NR and TA98, suggesting that these congeners are not acted upon by the "classical" nitroreductase which is missing in TA98NR (Mermelstein et al., 1981). Recently, the wild-type gene of the nitroreductase which is inactivated in TA98NR (known as the "classical" nitroreductase) has been cloned (Watanabe et al., 1987). Introduction of this gene into strains deficient in "classical" nitroreductase activity restored the mutagenic activity of 1-NP but had no effect on the mutagenicity of 1,8-DNP (Watanabe et al., 1987; Watanabe et al., 1989). The nitroreductase which acts upon 1,8-DNP and 1,6-DNP has not yet been isolated.

The mutagenic activities of 1,8-DNP and 1,6-DNP are reduced markedly in TA98/1,8-DNP$_6$ relative to TA98 (McCoy et al., 1981; 1983). Originally TA98/1,8-DNP$_6$ was believed to be deficient in a nitroreductase species distinct from the "classical" nitroreductase (McCoy et al., 1981; Rosenkranz, 1982). However, subsequent studies showed that introduction of metabolites such as the nitroso derivatives did not restore the mutagenic response, indicating that

Table 4. Mutagenicity of nitropyrenes, and nitropyrene metabolites in the S. typhimurium strains TA98, TA98NR, and TA98/1,8DNP$_6$.

	TA98	TA98NR	TA98/1,8DNP$_6$	Reference
1-NP[a]	467	87	158	Tokiwa & Ohnishi, 1986
	700	60	700	Fifer et al., 1986
	647	116	396	Bryant et al., 1984
	453	35	ND	Howard et al., 1987
	339	10	428	Ball et al., 1984b
1-NOP	27000	26000	26000	Fifer et al., 1986
	2130	1480	ND	Howard et al., 1987
1,3-DNP	130000	16900	2440	Tokiwa & Ohnishi, 1986
	145000	24750	ND	Howard et al., 1987
1,6-DNP	255000	209000	32000	Fifer et al., 1986
	175000	96000	33000	Tokiwa & Ohnishi, 1986
	184000	191000	ND	Howard et al., 1987
1,6-NONP	34000	33000	9000	Fifer et al., 1986
1,6-ANP	10	4.4	ND	Howard et al., 1987
1,8-DNP	734000	401000	8000	Fifer et al., 1986
	205000	203000	3000	Bryant et al., 1986
	254000	264000	ND	Howard et al., 1987
	257000	215000	8590	Tokiwa & Ohnishi, 1986
1,8-NONP	82000	103000	2000	Fifer et al., 1986
1,8-ANP	55	22	ND	Howard et al., 1987
1,8-DAP	0	0	ND	Howard et al., 1987
1,3,6-TNP	40700	36600	25600	Rosenkranz & Mermelstein, 1983
	36985	78200	25200	Tokiwa & Ohnishi, 1986
1,3,6,8-TNP	15500	10000	14000	Rosenkranz & Mermelstein, 1983
	15211	17200	7090	Tokiwa & Ohnishi, 1986

The values given are revertants per nanomole.

[a] Abbreviations: 1-NP, 1-nitropyrene; 1-NOP, 1-nitrosopyrene; 1,3-DNP, 1,3-dinitropyrene; 1,6-DNP, 1,6-dinitropyrene; 1,6-NONP, 1-nitroso-6-nitropyrene; 1,6-ANP, 1-amino-6-nitropyrene; 1,8-DNP, 1,8-dinitropyrene; 1,8-NONP, 1-nitroso-8-nitropyrene; 1,8-ANP, 1-amino-8-nitropyrene; 1,8-DAP, 1,8-diaminopyrene; 1,3,6-TNP, 1,3,6-trinitropyrene; 1,3,6,8-TNP, 1,3,6,8-tetranitropyrene.

the block in metabolism was not at the rate limiting (nitro to nitroso) step in nitroreduction. It is now known that TA98 /1,8-DNP_6 is deficient in acetyltransferase activity (McCoy et al., 1983, Orr et al., 1985). The gene encoding the functional acetyltransferase has also been cloned (Watanabe et al., 1987). When plasmids harboring this gene were introduced into an acetyltransferase deficient strain the mutagenic activity of 1,8-DNP was increased by a factor of 2000 (Watanabe et al., 1987). Although N-acetylated metabolites of 1,8-DNP have been isolated, it is unlikely that these derivatives are mutagenic. Instead, it is believed that the acetyltransferase also catalyzes the formation of unstable and extremely reactive N-acetoxy derivatives of 1,8-DNP and 1,6-DNP. It has been postulated that metabolic activation of 1,6-DNP and 1,8-DNP is facilitated by a tightly coupled nitroreduction/O-acetylation enzymatic activities (Howard et al., 1987).

Multiple nitroreductase species are also present in E. coli and Bacteroides fragilis (reviewed in McCalla, 1983; Kinouchi and Ohnishi, 1983). However, the activity of these enzymes towards nitropyrenes have not been extensively investigated. Likewise, the role of acetylation in the metabolism of nitropyrenes has not been investigated in bacterial strains other than S. typhimurium.

1.2.2 DNA Adduct Formation in Bacteria

The structure of the major DNA adducts formed by 1-NP and 1,8-DNP in S. typhimurium support the notion that reductive metabolism of a nitro group is responsible for activation of these chemicals to mutagenic species. Beland and coworkers used mammalian

xanthine oxidase to catalyze the reaction between 1-NP and calf thymus DNA (Howard et al., 1982; 1983). This reaction is optimal at pH 5 in an argon purged solution. The pH dependence for DNA adduct formation is highly indicative of an hydroxylamine intermediate (Kadlubar and Beland, 1985). The identity of the major adduct formed was N-(2'-deoxyguanosin-8-yl)-1-aminopyrene (dG-C(8)-AP). This adduct was identical to the major DNA adduct formed when 1-NP was incubated with S. typhimurium TA1538. There was a strong correlation between the extent of dG-C(8)-AP formation and the frequency of induced mutations in this strain (Howard et al., 1983). When 1-NOP, a partially reduced derivative of 1-NP, was further reduced to N-hydroxy-1-aminopyrene with ascorbate, and then reacted with calf thymus DNA at pH 5.0, dG-C(8)-AP was formed; the adduct was identical to the adduct formed in S. typhimurium by 1-NOP (Heflich et al., 1985). Together these results suggest that reduction of the nitro moiety of 1-NP, first to a nitroso group and then to an unstable hydroxylamine derivative, yields a metabolite which reacts with DNA.

The major DNA adduct formed by 1,8-DNP in S. typhimurium has also been characterized. Andrews et al. (1986) showed that ascorbate reduction of 1-nitroso-8-nitropyrene (1,8-NONP) to N-hydroxyl-1-amino-8-nitropyrene (1,8-HANP), followed by in vitro reaction of 1,8-HANP with calf thymus DNA at pH 5.0 yielded a single major DNA adduct. This adduct was identified as N-(2'-deoxyguanosin-8-yl)-1-amino-8-nitropyrene (dG-C(8)-ANP) (Figure 3). When S. typhimurium was exposed to tritiated 1,8-DNP, a single DNA adduct was detected which co-chromatographed with dG-C(8)-ANP. Degradation of the S.

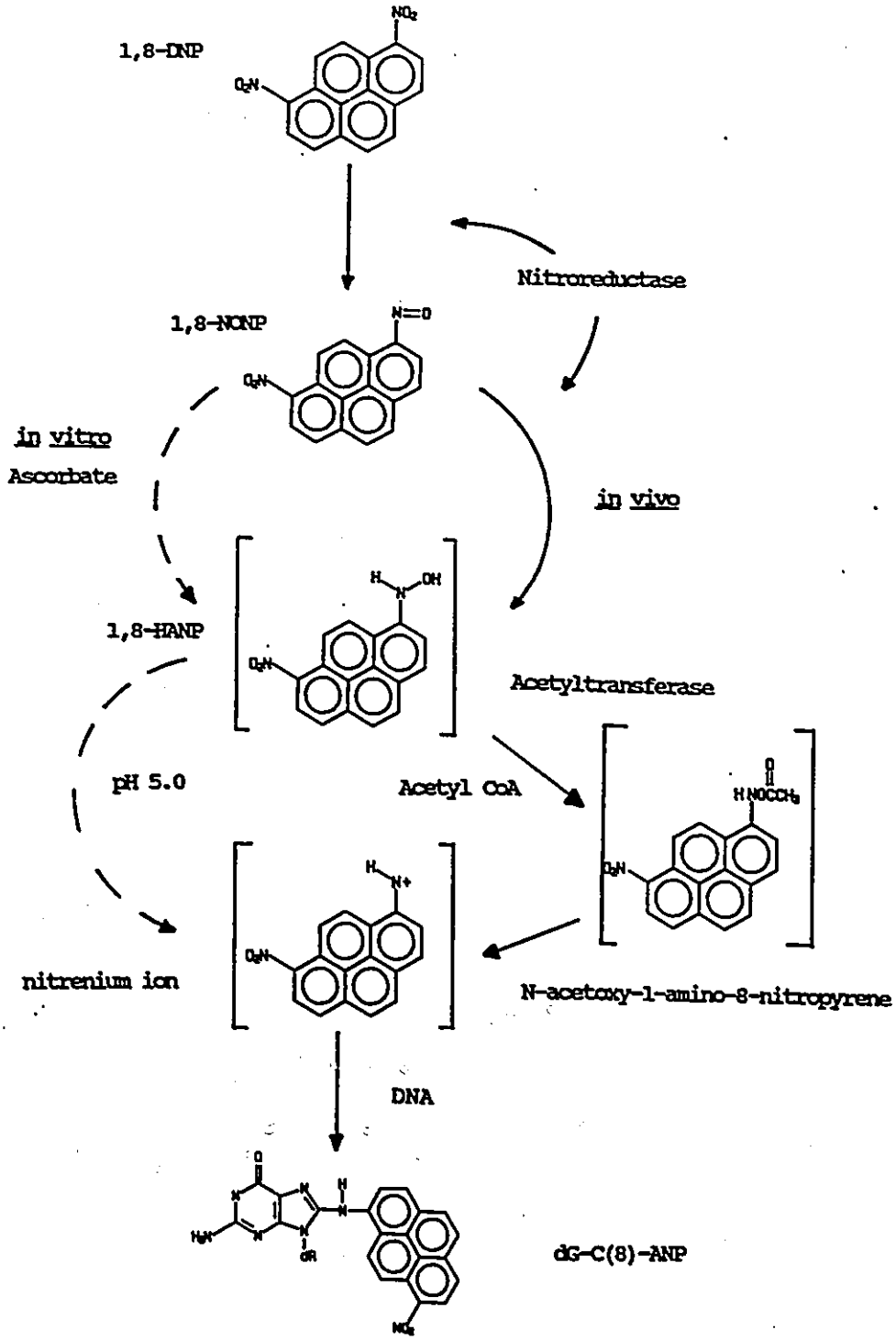


Figure 3. DNA adduct formation by 1,8-DNP in bacteria.
The pathway given by the broken line (- - - -) is for DNA adduct formation in vitro as described by Andrews et al. (1986).

typhimurium adduct with either acid or base yielded breakdown products which were chromatographically identical to those produced when dG-C(8)-ANP was treated in a similar fashion (Andrews et al., 1986; Andrews, 1988). This evidence suggests that: 1) the DNA adducts formed in vitro with 1,8-HANP, and in S. typhimurium exposed to 1,8-DNP are the same; and 2) the DNA lesion formed in bacteria exposed to dinitropyrene is produced via reductive metabolism of a single nitro group.

1.2.3 Metabolism and DNA Adduct Formation in Mammals

Nitropyrenes are metabolized in mammalian cells by both oxidative and reductive pathways. Several microsomal (NADPH-cytochrome P-450 reductase) and cytosolic (xanthine oxidase, aldehyde oxidase, and DT-diaphorase) enzymes (McCalla, 1983; Tatsumi et al., 1986; Tee et al., 1988) have nitroreductase activity which is often inhibited by oxygen (McCalla, 1983). Ring hydroxylation, which is catalyzed principally by microsomal cytochrome P-450 isozymes (Howard et al., 1988), requires the presence of oxygen. In the whole animal, the presence of intestinal microflora, which contain nitroreductases, provide an added level of complexity.

The metabolites observed when 1-NP was incubated with rat liver S9 were 3-hydroxy-1-nitropyrene, 6-hydroxy-1-nitropyrene, 8-hydroxy-1-nitropyrene, 4,5,-dihydro-4,5-dihydroxy-1-nitropyrene (formed through 1-nitropyrene-4,5-oxide) and small amounts of 1-aminopyrene (El-Bayoumy and Hecht, 1983). When 1-NP was administered to F344 rats by gavage, intravenous injection, or i.p. injection, these same products or their conjugates were recovered as fecal and

urinary metabolites (El-Bayoumy et al., 1983; Ball et al., 1984a; Howard et al., 1985). In addition to the hydroxylated metabolites, 1-amino-6-hydroxypyrene, 1-amino-8-hydroxypyrene, N-acetylamino-hydroxypyrenes (NAAP), and 1-aminopyrene were identified in the metabolite mixture recovered from the F344 rats (El-Bayoumy et al., 1983; Ball et al., 1984a). The reduced metabolites were not recovered from germfree F344 rats treated by gavage, suggesting that the intestinal microflora contribute to the reductive metabolism of 1-NP (El-Bayoumy et al., 1983; 1984b). Isolated rat trachea, alveolar macrophages and lung tissue, and cultured rat tracheal epithelial cells were extremely active in metabolizing 1-NP; both ring hydroxylation and nitroreduction products were found in significant quantities (Bond and Mauderly, 1984; Bond et al., 1985; 1986; King et al., 1986; 1987). 10-Hydroxy-1-nitropyrene was found to be a unique metabolite of respiratory tissue (Bond and Mauderly, 1984; King et al., 1986). A number of the 1-NP metabolites formed in mammalian cells (3-hydroxy-1-nitropyrene, 10-hydroxy-1-nitropyrene, NAAP, and 1-nitropyrene-4,5-oxide) have been shown to be mutagenic in S. typhimurium tester strains (Ball et al., 1984b; Ohnishi et al., 1986, King et al., 1987).

Several studies have examined DNA adduct formation by 1-NP in mammalian cells. The dG-C(8)-AP adduct has been detected in: 1) the liver, lung and kidney of $B6C3F_1$ mice following intratracheal instillation (Mitchell, 1988); 2) the lung and liver of newborn mice following i.p. injection (El-Bayoumy et al., 1988b); 3) the liver, kidney, and mammary glands of Wistar rats following i.p. injection

(Stanton et al., 1985); and 4) the mammary glands and livers of Sprague-Dawley rats treated with 1-NP by gavage (Roy et al., 1989). In all of these studies a significant amount of additional 1-NP derived material, which was chromatographically distinct from the dG-C(8)-AP adduct, was also bound to DNA. It is likely that the additional DNA adducts result from ring-oxidation of 1-NP (Roy et al., 1989).

In contrast to 1-NP, there is no evidence that 1,6-DNP or 1,8-DNP are metabolized by oxidative pathways in mammalian cells. Both in vivo (Heflich et al., 1986a) and in vitro (Djuric et al., 1985; 1986; 1987; King, 1988; Tee et al., 1988) studies suggest that metabolism of 1,6-DNP and 1,8-DNP occurs principally by reduction of the nitro groups to amino derivatives followed by N-acetylation. The principal metabolites of 1,8-DNP recovered from these studies were 1-nitroso-8-nitropyrene, 1-amino-8-nitropyrene, 1-acetylamino-8-nitropyrene, 1,8-diaminopyrene, 1-acetylamino-8-aminopyrene, and 1,8-diacetylaminopyrene (Djuric et al., 1985; Heflich et al., 1986a; Tee et al., 1988; King, 1988).

Beland and coworkers used rat and dog liver cytosols to study 1,6-DNP and 1,8-DNP metabolism and DNA adduct formation in vivo; the results of their experiments suggested that DNA adduct formation required both reduction and O-acetylation (Djuric et al., 1985; 1988). The identity of the DNA adducts recovered following i.p. treatment of rodents with 1,6-DNP or 1,8-DNP is also consistent with the notion that reductive metabolism is important for DNA adduct formation. The principal adduct formed by 1,6-DNP in the livers of

preweanling mice (Delclos et al., 1987a), and in the liver, kidney, bladder, and mammary gland of Sprague Dawley rats (Djuric et al., 1988) following i.p. injection is the dG-C(8) adduct, N-(2'-deoxyguanosin-8-yl)-1-amino-6-nitropyrene. Similarly, ^{32}P-postlabelling of the DNA adducts formed following i.p. injection of female CD rats with 1,8-DNP showed that a single major adduct was formed in the mammary glands, mesentery tissue, lung, and kidney; the adduct was chromatographically identical to the 3',[^{32}P]5'-diphosphate derivative of the dG-C(8)-ANP adduct described by Andrews et al. (1986) (Norman, 1988). Treatment of rat trachea epithelial cells with either 1,8-DNP or 1,8-NONP also resulted in the formation of dG-C(8)-ANP (Norman et al., 1989a).

1.3 Correlates Between Metabolism, DNA Adduct Formation, and Genotoxicity of Nitropyrenes

The genotoxic potency of nitropyrenes appears to be related to the mechanism of metabolism (Djuric et al., 1985; Eddy et al., 1986; Howard et al., 1987). In bacteria, 1,6-DNP and 1,8-DNP are metabolized to reactive species by nitroreduction (2 electron transfer) followed by acetylation. On the basis of the evidence that is presently available, 1,6-DNP and 1,8-DNP are metabolized in a similar manner in mammalian cells. These two congeners are mutagenic in both bacterial and mammalian cells (Tables 1 & 2), and are also potent carcinogens in experimental animals (Table 3). The major DNA adduct which is formed by 1,8-DNP and 1,6-DNP in both bacterial and mammalian cells in culture, and rodents, is the dG-C(8) adduct (Andrews et al., 1986; Delclos et al., 1987a; Djuric et al., 1988;

Norman, 1988; Norman et al., 1989a). The dG-C(8)-ANP lesion was present at the highest levels in the mammary gland and the mesentery tissue following a single i.p. injection of 1,8-DNP in rodents (Norman, 1988). This correlates with the sites at which tumors are most frequently induced by this chemical (King, 1988). Thus, the bulk of the evidence presently available is consistent with reductive metabolism of 1,8-DNP and 1,6-DNP to species which react at the C(8) position of guanine to yield a DNA adduct which is biologically active.

In relative terms, 1-NP and 1,3-DNP are less mutagenic than the 1,6-DNP and 1,8-DNP congeners (Tables 1 and 2), and are much less carcinogenic (Table 3) (King, 1988). It is believed that 1-NP and 1,3-DNP are reduced by single electron transfers at the rate determining step of nitroreduction (Klopman et al., 1984; Howard et al., 1987). Nitroreduction of 1-NP and 1,3-DNP in S. typhimurium is mediated by the "classical" nitroreductase, which is different from the enzyme which reduces 1,6-DNP and 1,8-DNP. 1-NP and 1,3-DNP are not easily reduced in mammalian cells: 1-NP is metabolized largely by ring hydroxylation; and 1,3-DNP is reduced very inefficiently by the cytosolic nitroreductases which act on 1,6-DNP and 1,8-DNP (King, 1988).

1.4 Objectives of this Research

It is clear that DNA is a principal target for the biological activity of nitropyrenes. However, relatively little is known about the mechanisms by which these compounds induce mutations. In order to evaluate nitropyrene-induced mutagenesis more completely, it is

necessary to obtain detailed information regarding the premutational lesions which are formed by these compounds, the types of mutations which are induced, and the sites at which they occur. The experiments described in this thesis have used two extremely sensitive experimental systems, the ^{32}P-postlabelling technique and the lacI system of E. coli, to study 1,8-NONP mutagenesis in E. coli. The objectives of these studies relate to two distinct areas of research: the first is the nature of the DNA adducts formed by 1,8-NONP; the second is the nature of the DNA sequence changes induced in E. coli by 1,8-NONP. These are described in more detail below.

1.4.1 DNA Adduct Formation by 1,8-NONP

A variety of evidence has demonstrated that in both bacteria and mammalian cells 1,8-DNP is metabolized (via 1,8-NONP, 1,8-HANP, and N-acetoxy-1-amino-8-nitropyrene) to yield a nitrenium ion which reacts with DNA. Although the principal site of reaction of the nitrenium ion in DNA is the C(8) position of guanine, it is possible that minor products which might be of significance to mutation are also formed. Previous methods used for analysis of DNA adducts formed by 1,8-DNP, and reduced derivatives of 1,8-DNP (Andrews et al., 1986), are probably too insensitive to have detected minor adducts. A second disadvantage of these methods is that radiolabelled compounds are required in order to detect DNA adducts formed in vivo. The recent development of an ultrasensitive ^{32}P-postlabelling technique (Randerath et al., 1981; Gupta et al., 1982; Reddy et al., 1984; Gupta, 1985; Reddy and Randerath, 1986) provides a means of detecting DNA adducts at very low levels without the use

of radiolabelled mutagens. Application of the ^{32}P-postlabelling technique to the analysis of 1,8-NONP-modified DNA is described in Chapter 2. The principal objectives of the experiments described in Chapter 2 were: 1) to determine the postlabelling pattern of DNA adducts produced by 1,8-NONP in E. coli; 2) to compare the DNA adducts formed by 1,8-NONP in E. coli with those produced by the in vitro reaction of 1,8-HANP with DNA; and 3) to examine the properties of any minor products which might be detected.

1.4.2 Characterization of the mutations induced by 1,8-NONP

Very little is known regarding the mutational specificity (i.e. the types of mutations induced, and the sites at which the mutations occur) of nitropyrenes. Such knowledge would be useful in assessing mutational mechanisms, particularly if supplemented with studies describing the influence of DNA repair background on the nature of the induced mutations. Prior to the work described in this thesis there have been no studies which have examined forward mutations induced by 1,8-DNP or its partially reduced derivative 1,8-NONP at the DNA sequence level. Chapter 3 of this thesis provides a detailed description of the DNA sequence changes induced in the lacI gene of E. coli by 1,8-NONP, and discusses possible inferences of the results with regard to mechanism of mutation. The objectives for the experiments described in this chapter were: 1) to determine the mutational specificity of 1,8-NONP; 2) to evaluate the influence of plasmid pKM101 on the mutational spectrum; and 3) to determine the influence of excision repair on 1,8-NONP mutagenesis.

2. ^{32}P-POSTLABELLING OF DNA ADDUCTS FORMED BY 1,8-NONP

2.1 INTRODUCTION

2.1.1 Methods for the Detection of DNA adducts

More than 25 years ago Brookes and Lawley (1964) demonstrated that a positive correlation exists between the mutagenic and carcinogenic potency of some chemicals and the extent to which they bind covalently to DNA. This relationship has provided impetus for the development of sensitive methods which facilitate the detection and characterization of DNA lesions. Valuable information can be obtained through the application of such methods. In the area of human toxicology, DNA adducts can help identify potential carcinogens in environmental samples (Reddy and Randerath, 1986), and provide information of human exposure to known carcinogens (Harris et al., 1985). Insight regarding mechanisms of induced mutation and DNA repair can be obtained by identification and quantitation of DNA adducts. In addition, the ability to monitor formation and repair of DNA adducts within different tissues can be useful in studies regarding chemical carcinogenesis (Kriek et al., 1984).

Several factors complicate the study of chemical-DNA interactions. 1) Techniques must be capable of distinguishing between a lesion and unmodified bases when the latter are more numerous by many orders of magnitude. Detection and subsequent identification is complicated by the inherent instability of certain DNA adducts both within the cell and during analysis. 2) Most

nitrogen and oxygen atoms of nucleic acid bases, C(8) of purines, and phosphate groups of the nucleotides are potential sites of modification. Moreover, some DNA damaging agents have several activated forms. Consequently, a wide range of adduct structures can potentially be formed by a single chemical. Analysis of the multitude of DNA adducts formed by the components of complex mixtures presents an even greater challenge.

Studies of the interaction of chemicals with DNA have frequently used radiolabelled compounds to aid in detection, quantitation, and identification of DNA adducts. This type of methodology is particularly useful when high resolution chromatographic techniques and synthetic reference compounds are available to facilitate identification (Kriek et al., 1984). However, studies of this nature have several limitations: relatively few carcinogens or mutagens are available in an isotopically labelled form; the sensitivity with which adducts can be detected is generally limited by the low specific activity of the available radiolabelled compounds; and ethical considerations obviously limit in vivo studies to non-human subjects. In light of these restrictions, a great deal of research has examined alternative methods which overcome at least some of these constraints (Maugh, 1984). Three very different techniques have been shown to be particularly useful: monoclonal antibodies; fluorescence spectrophotometry; and ^{32}P-postlabelling. These are discussed below.

Monoclonal antibodies have been produced against a wide range of DNA adducts (Baan et al., 1985), including those formed by

polyaromatic hydrocarbons (PAHs) such as benzo(a)pyrene (Santella et al., 1985; Harris et al., 1985), alkylating agents (Muller and Rajewsky, 1981; Adamkiewicz et al., 1985) and cisplatin (Baan et al., 1985). A variety of competitive radioimmunoassays or solid phase assays allow accurate determination of the degree of DNA modification to a level of about one adduct in 10^7 to 10^8 nucleotides (Muller and Rajewsky, 1981; Poirier, 1981; Kriek et al., 1984). In addition, development of immunofluorescent microscopic techniques allows adduct analysis in intact cells (Baan et al., 1985). The technique is, however, limited by cross-reactivity with similar adducts, and by the requirement that it must be possible to synthesize the adduct, and attach it to a carrier protein in order to obtain a highly antigenic form (Muller and Rajewsky, 1981; Kriek et al., 1984).

Adducts formed with fluorescent compounds can be detected by a variety of spectrophotometric techniques (Kriek et al., 1984; Vahakangas et al., 1985a). Recently, adducts formed with benzo(a)pyrene-diolepoxide (BPDE) (Vahakangas et al., 1985a; 1985b) and aflatoxin B_1 (AFB_1) (Harris et al., 1986) have been detected at a level of about one adduct in 10^7 nucleotides by synchronous fluorescence spectrophotometry (SFS). General use of these techniques is limited for several reasons: 1) the fact that many adducts have similar fluorescence spectra imposes constraints on both identification and quantitation; 2) fluorescence detection of the low levels of DNA adducts in biologically relevant samples is subject to quenching by DNA; 3) highly fluorescent compounds comprise only a fraction of environmental carcinogens and mutagens; and 4) methods

such as SFS require sophisticated instrumentation which limits its widespread use as a standard laboratory technique (Kriek et al., 1984; Vahakangas et al., 1985a).

2.1.1.1 ^{32}P-Postlabelling of DNA adducts

Randerath and his associates have developed an extremely sensitive postlabelling technique which can be used to detect DNA adducts formed by most DNA damaging agents (Randerath et al., 1981; Gupta et al., 1982; Reddy et al., 1984; Gupta, 1985; Reddy and Randerath, 1986). The standard assay (Randerath et al., 1981; Gupta et al., 1982) contains five major steps (Figure 4): 1) modified DNA is digested to 3'-phosphodeoxyribonucleosides using micrococcal nuclease and spleen phosphodiesterase; 2) a ^{32}P-labelled phosphate group is incorporated onto the 5'-hydroxyl of 3'-phosphodeoxyribonucleosides by phage T4 polynucleotide kinase (PNK) to form 3',[^{32}P]5'-diphosphodeoxyribonucleosides; 3) labelled normal nucleotides are separated from labelled adducted nucleotides by polyethyleneimine (PEI)-cellulose thin layer chromatography (TLC); 4) the DNA adducts are resolved in two dimensions by PEI-cellulose TLC to produce a fingerprint pattern; and 5) the labelled adduct nucleotides are detected by autoradiography and quantitated by Cerenkov assay (Randerath et al., 1984). A significant disadvantage of this protocol is that the entire DNA digest (containing both normal and modified nucleotides) must be phosphorylated with ATP ([γ-^{32}P]ATP + unlabelled carrier). This means that the most of the radioactive ATP is consumed by labelling the normal nucleotides which vastly outnumber adducted nucleotides. Consequently, the limit of

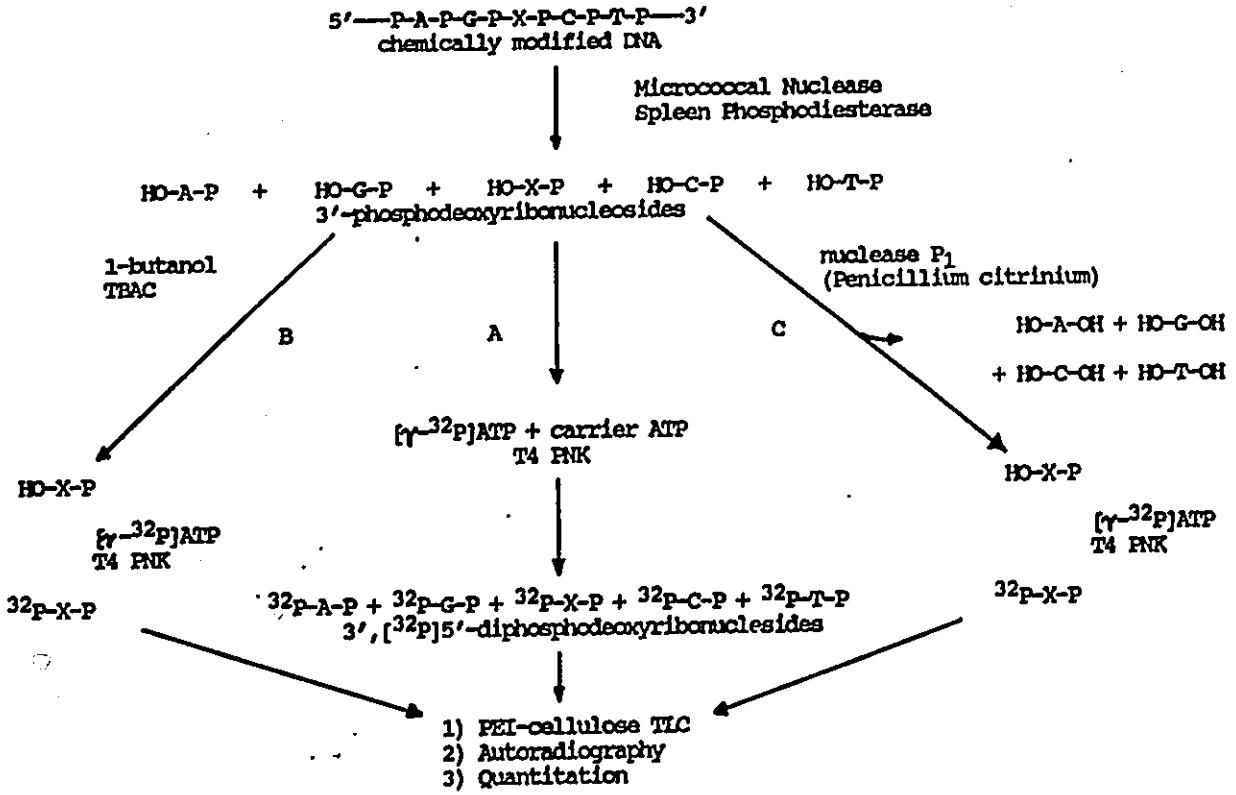


Figure 4. Techniques for ^{32}P-postlabelling DNA adducts. A) The standard postlabelling technique (Randerath et al., 1981; Gupta et al., 1982). Normal and adducted 3'-phosphodeoxyribonucleosides are labelled in the presence of [γ-^{32}P-ATP and excess carrier ATP. B) The butanol extraction procedure as described by Gupta (1985). TBAC is the phase transfer reagent tetrabutylammonium chloride. DNA adducts are enriched by extraction into 1-butanol in the presence of TBAC prior to labelling with carrier-free [γ-^{32}P]-ATP. C) The nuclease P_1 method as described by Reddy and Randerath (1986). In this procedure the normal 3'-phosphodeoxyribonucleosides are dephosphorylated by nuclease P_1. The adduct nucleotides, which are resistant to nuclease P_1, are then labelled with carrier-free [γ-^{32}P]ATP. In this diagram A is deoxyadenosine; G is deoxyguanosine; C is deoxycytidine; T is deoxythymidine; X is an adduct deoxyribonucleoside; P is phosphate.

detection is only about 1 adduct in 10^6-10^7 nucleotides (Gupta et al., 1982; Gupta, 1985). The development of methods which eliminate unmodified 3'-phosphodeoxyribonucleosides prior to the labelling step (Gupta, 1985; Reddy and Randerath, 1986) has greatly increased the sensitivity of the procedure. Separation of the unmodified nucleotides makes it possible to label the DNA adducts with excess carrier-free [γ-^{32}P]ATP; this results in an increase in sensitivity of about 3 orders of magnitude.

Two different strategies have been used to separate adducts from unmodified nucleotides (Figure 4). Gupta (1985) showed that nonpolar adducts in a DNA digest could be physically separated from normal nucleotides by extraction with 1-butanol in the presence of the phase transfer agent tetrabutylammonium chloride (TBAC); the modified nucleotides partition extremely efficiently into the butanol phase, while the normal ones remain in the aqueous phase. In the second method, the substrate specificity of nuclease P_1 has been used to advantage (Reddy and Randerath, 1986). Nuclease P_1 efficiently dephosphorylates unmodified 3'-phosphodeoxyribonucleosides but does not recognize most adducted 3'-phosphodeoxyribonucleosides. Since the unphosphorylated deoxyribonucleosides are not substrates for PNK, treatment with nuclease P_1 provides a rather elegant means of concentrating the adducted 3'-phosphodeoxyribonucleosides. At the present time, most workers in the field use one of these two enhancement techniques.

The usefulness of this technique is attributable to several factors. The high specific activity of ^{32}P makes the procedures

extremely sensitive, with a limit of detection of one adduct in 10^{10} nucleotides when enhancement techniques are used (Gupta, 1985; Reddy and Randerath, 1986). Thus, it is possible to detect even minor adducts formed by some compounds. In addition, the technique can be used to detect adducts present in DNA at levels which arise from human exposures. The fact that radiolabelled compounds are not necessary makes this technique potentially useful for the detection of DNA lesions produced by many different compounds; therefore the method is ideal for studying DNA adducts formed by the complex mixtures which are encountered in the environment. Since the methodology lends itself to quantitation, information regarding total DNA adduct present, and the kinetics of DNA adduct formation and disappearance can be obtained (Gupta and Dighe, 1984; Norman et al., 1989a). Finally, DNA adduct formation can be assessed in as little as 1 µg of DNA, so that DNA alterations can be examined when relatively small biological samples are available.

The ^{32}P-postlabelling technique has been used in a variety of different studies which have served to verify its utility (reviewed in Watson, 1987). However, a number of disadvantages remain. 1) An ongoing problem is the difficulty involved in trying to identify the adducts which are detected. 2) Several studies have shown that an unexpectedly large number of "adducts" are recovered in DNA treated with chemicals that had previously been studied extensively using other methods (Gupta et al., 1982; Reddy et al., 1984, Randerath et al., 1984). It is often difficult to determine whether the additional products represent unique adducts, or artifacts. 3) Some

adducts are refractive to analysis using enhancement techniques. For instance, guanine residues modified at the C(8) position are often substrates for nuclease P_1 and therefore will not be detected using this technique (Gupta and Early, 1988). Consequently, DNA modification by aromatic amine and nitroaromatic compounds, which react most frequently at C(8) of guanine, is likely to be badly underestimated by the nuclease P_1 method. On the other hand, some polar adducts partition poorly into butanol, and consequently will be not be detected using the butanol extraction technique described by Gupta (1985).

2.1.2 Reaction of N-hydroxy Arylamines With DNA

Reduction of nitroarenes (Howard et al., 1983; Andrews et al., 1986) or oxidation of aromatic amines (Beland and Kadlubar, 1985) produces N-hydroxy arylamine derivatives which can be enzymatically or nonenzymatically converted to electrophilic species which react with DNA (reviewed in Kadlubar and Beland, 1985). Reaction of the arylhydroxylamine derivatives with DNA occurs very slowly at physiological pH (Kadlubar and Beland, 1985; Andrews et al., 1986). In mammalian cells, and also in S. typhimurium, formation of the ultimate mutagen is enhanced considerably by acetylation (section 1.2.1; King and Glowinski, 1983; Orr et al., 1985; Saito et al, 1985). It is believed that the mutagen which is derived from acetylation is an O-acetoxy arylamine species which would be expected to undergo heterolytic cleavage to form a partially delocalized nitrenium/carbenium ion electrophile as shown in Figure 5 (Kadlubar and Beland, 1985).

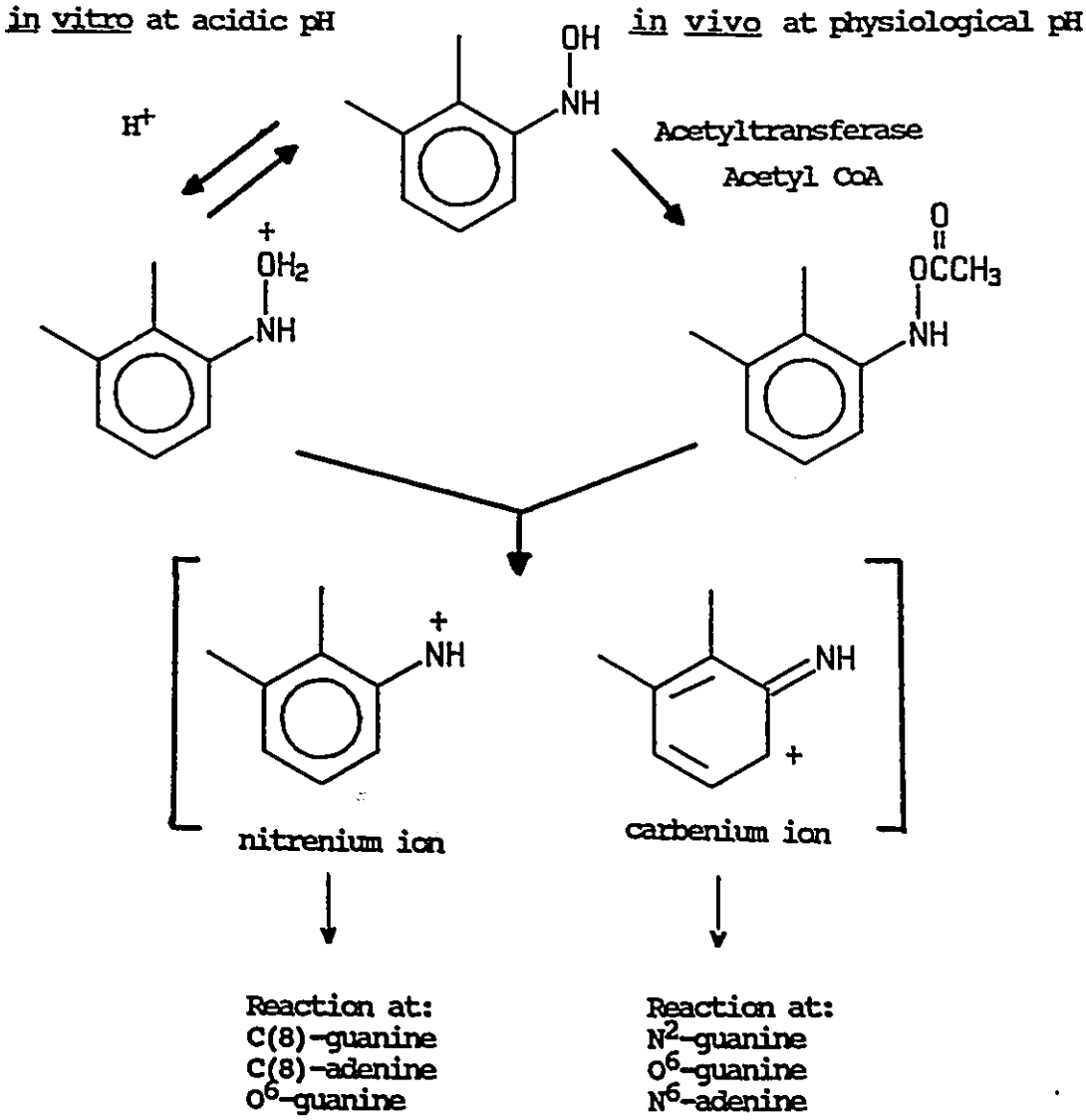


Figure 5. Reaction of N-hydroxy arylamines with DNA. A nitrenium/carbenium electrophile can be formed: A) nonenzymatically via protonation at acidic pH; or B) enzymatically via O-acetylation *in vivo*.

Reaction of N-hydroxy arylamine derivatives with DNA occurs readily at a slightly acidic pH. Protonation of the N-hydroxy group, which occurs readily between pH 5 and 6, followed by the elimination of water yields the reactive nitrenium/carbenium ion intermediate (Figure 5) (Kriek, 1965; Kadlubar et al, 1978). The direct reactivity of N-hydroxy arylamines compounds with DNA at acidic pH has made it possible to gather important information regarding DNA adducts using relatively simple in vitro experiments (reviewed in Beland et al, 1983; Beland and Kadlubar, 1985).

DNA adduct formation results from reaction of the nitrenium/carbenium electrophile with purine nucleophiles. The sites of modification for N-substitution are the C(8) positions of guanine and adenine, and the O^6 position of guanine; ortho-substitution occurs principally at the N^2 and O^6 positions of guanine and the N^6 position of adenine (see section 2.4 for detailed references).

2.1.3 Experimental Approach

The principal objective of the experiments described in this chapter was to use the ^{32}P-postlabelling method to detect DNA adducts formed with 1,8-NONP in E. coli. In order to obtain information regarding the nature of the adducts detected in E. coli, several postlabelling experiments were conducted with DNA (calf thymus, poly(dG.dC), and poly(dA.dT)) which had been modified in vitro with N-hydroxy-1-amino-8-nitropyrene (1,8-HANP) at pH 5.0. In the studies presented in this chapter, both the standard ^{32}P-postlabelling protocol (Randerath et al., 1981; Gupta et al, 1982) and the butanol enhancement technique (Gupta, 1985) have been used. The mutational

consequences of 1,8-NONP-DNA adducts in E. coli have been determined and are described in Chapter 3.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Succinic acid, $CaCl_2$, $MgCl_2.6H_2O$, 1-butanol, citric acid, phenol, formic Acid (98-100 %), LiOH, and LiCl were purchased from BDH Chemicals. Spermidine (free base), DL-dithiothreitol, adenosine 5'-triphosphate (disodium salt), micrococcal endonuclease (EC 3.1.31.1, grade VI, 0.21 U/µg), potato apyrase (EC 3.6.1.5, grade I, 0.002 U/µg), RNase A (EC 3.4.27.5, type III-A), RNase T_1 (EC 3.1.27.3), calf thymus DNA, polyethyleneimine (50%), and Bicine (N,N-bis(2-hydroxyethyl)glycine) were purchased from Sigma Chemical Co.. Electrophoresis grade urea and Tris were obtained from Biorad Laboratories. Chloroform and N,N-dimethylformamide were obtained from Caledon chemicals. [γ-^{32}P]ATP (3000Ci/mmol) was purchased from NEN Dupont. 2'-Deoxynucleoside 3',5'-diphosphates, alternating copolymers poly(dG.dC) and poly(dA.dT), and T4 polynucleotide kinase (EC 2.7.1.78) were purchased from Pharmacia (Canada) Inc.. Spleen phosphodiesterase (from calf spleen, EC 3.1.16.1, 0.002 U/µg) was obtained from Boeringer Mannheim. Concentrated ammonium hydroxide and HCl were purchased from J.T. Baker Chemical Co.. MN300 Cellulose was supplied by Brinkman. Planished vinyl sheets were purchased from Transilwrap of Canada (Scarborough, Ontario). Bactotryptone and yeast extract were obtained from Difco. The synthesis of 1,8-NONP (purity > 99 %) by oxidation of 1-amino-8-nitropyrene was previously described by Andrews *et al.* (1986).

E. coli strain NR6113 (*ara* Δ(*lacpro*) *thi* $Tsv^r(CV^S)$ F'*lacpro* Δ(*bioFCD-uvrB-chlA*)) was obtained from B.W. Glickman, York University,

Toronto, Ontario.

2.2.2 Methods

2.2.2.1 Preparation of PEI-Cellulose Sheets

Polyethyleneimine (PEI)-cellulose sheets were prepared as described by Randerath and Randerath (1964). A solution of 0.5 % PEI, pH 6.0 was prepared from a 50 % stock solution. A suspension of MN300 cellulose (15.2 g per 100 ml) in 0.5 % PEI was homogenized in a warring blender for about 45 seconds. Vinyl Transilwrap planished sheets (washed with methanol and water) were attached to thick glass plates and a 0.5 mm layer of the PEI-cellulose suspension spread evenly onto the plastic. After drying overnight at room temperature the sheets were cut to their final size (17 x 20 cm), a Whatman #1 wick attached and the sheets washed by ascending chromatography in distilled water overnight. The sheets were dried in air, wrapped in aluminum foil, and kept in the refrigerator until used.

2.2.2.3 Preparation of DNA adducts

In vitro. DNA adducts of reduced 1,8-dinitropyrene derivatives were prepared in vitro according to the methods outlined by Andrews et al. (1986). Solutions of calf thymus DNA (CT DNA) (1 mg/ml) or alternating copolymers poly(dG.dC) and poly (dA.dT) (0.5 mg/ml) were prepared in citrate buffer (20 mM, pH 5.0). Prior to reaction the solutions were made 25 % in dimethylformamide (DMF) and degassed with argon. Approximately 500 nmol of 1,8-NONP was dissolved in 50 ul of DMF and added to 125 µl of the DNA solution. Five molar equivalents of ascorbate were added to reduce the nitroso moiety to the hydroxylamine. The mixture was left for sixteen hours

in the dark at room temperature. Residual chemical was extracted from DNA with phenol:chloroform:isoamyl alcohol (25:24:1), and the DNA precipitated from the aqueous phase by the addition of 0.1 volume of 5 M NaCl and 2 volumes of 95 % alcohol, washed with 70 % ethanol and stored at -20°C.

In vivo. Fifty ml of an overnight culture of E. coli strain NR6113 were pelleted by centrifugation and resuspended in 10 mls of 1 X VB salts. Five hundred µl of a 0.2 mM solution of 1,8-NONP (dissolved in DMSO) was added to the cultures and the mixture incubated for 20 minutes at 37° C following which the cells were pelleted by centrifugation and placed on ice. The bacterial pellet was resuspended in extraction buffer (0.2 M Tris-HCl, 0.1 M LiCl, 25 mM EDTA, 1 % SDS, pH 7.4) and extracted successively with phenol (distilled and saturated with buffer), phenol:chloroform:isoamyl alcohol (25:24:1), and finally chloroform:isoamyl alcohol (24:1). After the addition of 0.1 volume of 5 M NaCl, DNA was precipitated by the addition of 2 volumes of 95 % ethanol (precooled to -20°C). The precipitate was washed twice with 70 % ethanol and dissolved in solution buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). Residual RNA was destroyed by incubation at 37° C with a mixture of RNase T_1 (50 units/ml) and RNase A (100 µg/ml) (Gupta, 1984). After extraction with chloroform:isoamyl alcohol, the DNA was precipitated as described above, dissolved in water and the DNA concentration estimated spectrophotometrically (20 A_{260} units per mg DNA) (Gupta et al., 1982).

2.2.2.3 Digestion of DNA

To digest control or carcinogen modified DNA to 3'-phosphodeoxyribonucleosides, 1 µg of DNA was incubated at 37°C for 0.5-4.5 hr (normally 4 hr) with 2.5 µg each of micrococcal nuclease and spleen phosphodiesterase (prepared by dialysis of commercially available product against distilled water) in a succinate buffer (20 mM succinate, 10 mM $CaCl_2$, pH 6.0) (Gupta et al., 1982).

2.2.2.4 The Standard ^{32}P-Postlabelling Technique

An appropriate amount of radiolabelled [γ-^{32}P]ATP (20-200 µCi) was added to a 0.5 ml Eppendorf tube and dried in a desiccator under vacuum. A 1.7 µl aliquot of the digest was added to the tube containing the radiolabelled ATP along with a 7.1 µl solution consisting of 1 µl Bicine buffer (0.1 M Bicine-NaOH, 0.1 M $MgCl_2$, 0.1 M dithiothreitol, 10 mM spermidine, pH 9.5), 0.5 µl polynucleotide kinase, and 5.6 µl 17.5 % glycerol in distilled water. The mixture was incubated for 15 minutes at 37° C at which time 1.2 µl of 0.5 mM non-radioactive ATP was added, and the incubation continued for a further 30 minutes. After the addition of 2 µl of a freshly prepared 1:1 mixture of 3',5'-diphosphodeoxyribonucleosides (4 µg/µl each of dpAp, dpTp, dpGp and dpCp) and potato apyrase (40 mU/µl) the mixture was incubated for a further 30 minutes at 37°C.

To check the efficiency of the labelling steps and to calculate the amount of label incorporated into normal nucleotides, an aliquot of the labelled digest was diluted in distilled water and a fraction of this applied to PEI-cellulose and developed in 0.27 M ammonium sulfate to separate the labelled nucleotides.

The remaining labelled digest was applied at the origin of a 17 x 20 cm sheet of PEI-cellulose (see Figure 6). Development in direction 1 (D_1) was with 1.1 M LiCl or 1 M sodium phosphate pH 6.8. A Whatman #1 wick was stapled to the PEI sheet and allowed to extend out of the chromatography tank in order to achieve continual overnight development. Following development in D_1, the PEI sheet was cut 12 cm from the bottom of the sheet and the upper portion and wick, containing most of the radioactivity associated with normal nucleotides, was discarded. The remainder was washed with water, dried, and developed in direction 2 (D_2) to the top of the sheet with 2.5 M ammonium formate, pH 3.5. The chromatogram was again cut 12 cm from the bottom, and the upper portion was discarded. The remaining 12 x 12 cm sheet, containing the adduct nucleotides at the origin, was then washed with water and dried under warm air.

Resolution of the DNA adducts was achieved by 2 dimensional chromatography using high concentrations of urea. Optimal urea concentration for the resolution of the adducts examined in these studies was found to be 7.0 M. Development in direction 3 (D_3) was with a 3.0 M lithium formate solution, pH 3.5, 7.0 M urea, and direction 4 (D_4) was with 0.8 M LiCl, 0.5 M Tris-HCl, 7.0 M or 8.5 M urea, pH 8.0. A final wash of the chromatogram with high electrolyte solutions (0.35 M $MgCl_2$ or 1 M sodium phosphate, pH 6.8) was in "direction 5" (D_5-which runs in the same direction as D_4) following the attachment of a Whatman #1 wick. This last step was to remove any residual non-adduct radioactivity that might increase the background. Following development in D_5 the wick was removed, the

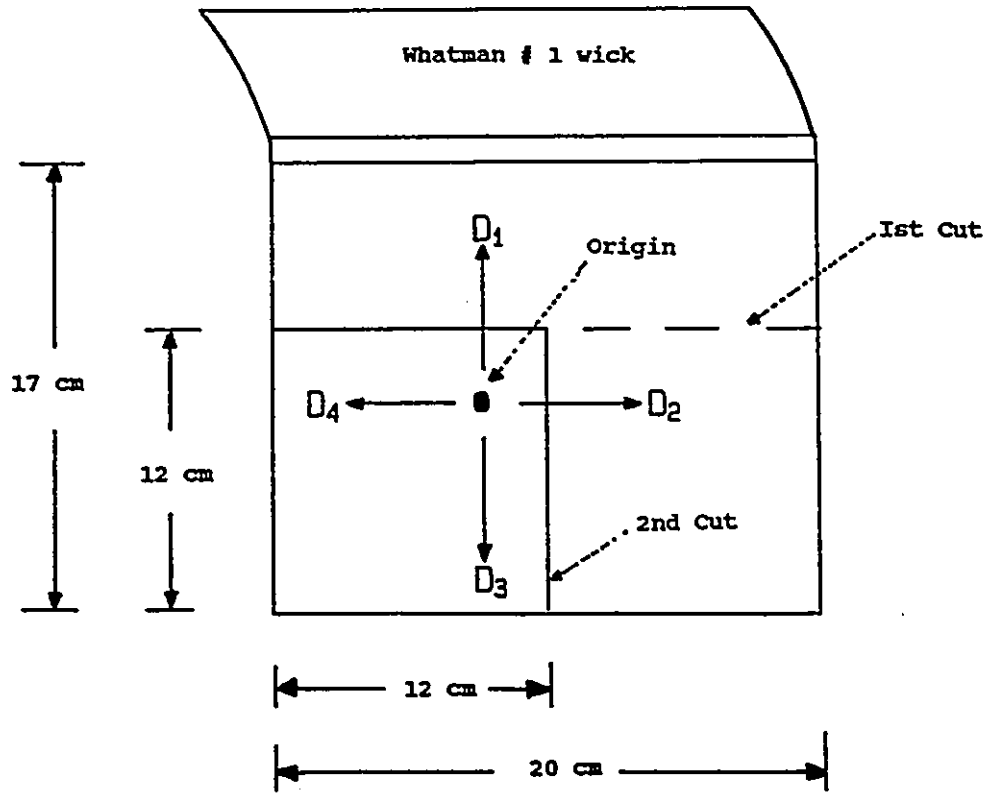


Figure 6. The TLC procedure used in the ^{32}P-postlabelling technique.
Sheets of PEI cellulose (17 x 20 cm) were marked as shown in this figure. A Whatman # 1 wick was stapled to the top of the sheet. The labelled DNA digest was spotted onto the origin. Following overnight development in D_1 the sheet was cut on the line marked "first cut" and the top portion of the PEI-cellulose sheet containing the wick was discarded. Following development in D_2, the sheet was cut along the line marked "second cut" and the right portion of the PEI cellulose sheet was discarded. The labelled DNA adducts did not move from the origin when the D_1 and D_2 solvents were used. The remaining 12 x 12 cm sheet was developed in D_3 and D_4 to resolve the DNA adducts into a fingerprint pattern. In Figures 8 to 12 the chromatograms have been rotated through 180° such that the origin is in the bottom left corner: D_3 running in the vertical direction, and D_4 running horizontally to the right.

sheet washed in distilled water and dried under a hair dryer.

2.2.2.5 The Butanol Extraction Method for ^{32}P-Postlabelling

The hydrophobic adducts were separated from the normal nucleotides in the following manner. Fifty µl of DNA digest (5 µg DNA) was mixed with 17.5 µl each of 100 mM ammonium formate (pH 3.5) and 10 mM TBAC in a total volume of 175 µl. The mixture was extracted twice with an equal volume of 1-butanol. The phases were separated by centrifugation in an Eppendorf centrifuge. The pooled butanol phases containing the DNA adducts were back-extracted twice with an equal volume of water, and then dried in the vacufuge. The combination of butanol extraction and back-extraction with water results in almost complete removal of normal nucleotides without significant loss of adducts (Gupta, 1985).

A "labelling mixture" was made up of 4 µl [γ-^{32}P]ATP (3000 Ci/mmol), 2.5 µl of 3X Bicine buffer (0.3 M Bicine, 0.1 M $MgCl_2$, 0.1 M DTT, 10 mM spermidine pH 9.5), 1 µl PNK and 0.5 µl water. The dried butanol extract was taken up in 10 µl 100 mM Tris-HCl, pH 9.5, added to 5 µl of the labelling mixture, and incubated for 30 minutes at 37^{o} C. To ensure that the adducts were labelled under conditions of excess ATP, 1 µl of this solution was removed, diluted 100-fold in water and spotted (3 µl) onto a PEI-cellulose strip. The strip was developed with 4.5 M ammonium formate, pH 3.5, a solution which clearly separates radiolabelled ATP from adducted nucleotides, residual normal nucleotides and inorganic phosphate (Figure 7C). The remainder of the labelled adduct solution was spotted onto a sheet of PEI-cellulose, and the adducts resolved using the 4-dimensional

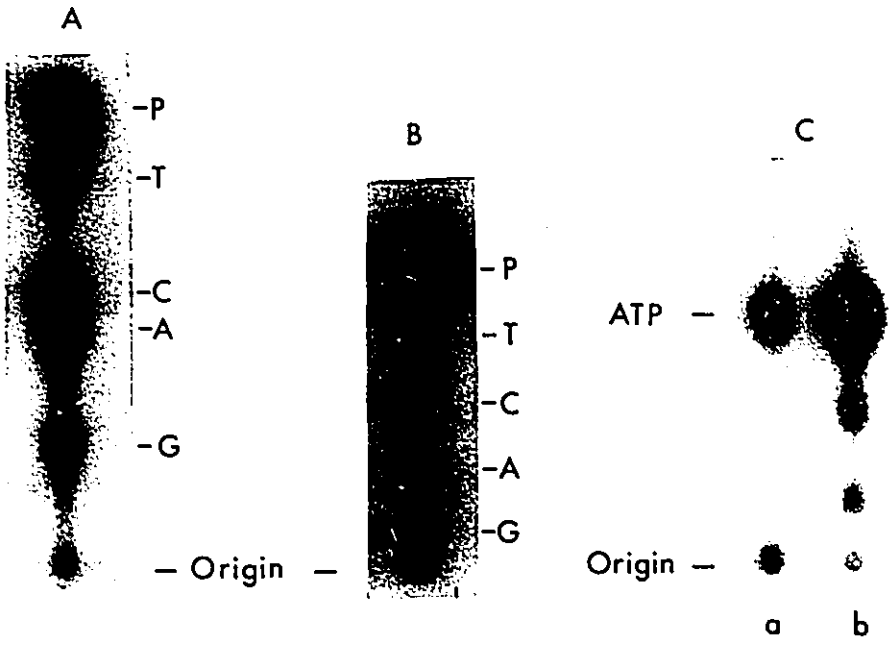


Figure 7. Ascending PEI-cellulose TLC of ^{32}P-labelled nucleotides. Panel A: total normal nucleotides were labelled with carrier-free [γ-^{32}P]ATP and developed with 0.27 M ammonium sulfate. Panel B: total normal nucleotides were labelled with carrier-free [γ-^{32}P]ATP and developed with 0.8 M Ammonium formate, pH 3.5. Panel C: diluted [γ-^{32}P]ATP (lane a) and 1-butanol-extracted, ^{32}P-labelled DNA adducts (lane b) were spotted onto PEI-cellulose and developed in 4.5 M ammonium formate, pH 3.5.

procedure detailed above, except that D_2 was omitted.

The total nucleotides were also assessed under conditions of excess [γ-^{32}P]ATP. One ng of digest was mixed with 2.5 µl of the labelling mix in a total volume of 7.5 µl. After incubation for 30 minutes at 37° C, 2 µl of a 1:1 mixture of 3',5'-diphosphodeoxynucleosides (4 µg/µl each of dpAp, dpTp, dpGp and dpCp) and potato apyrase (40 mU/µl) were added and the incubation was continued for 30 minutes. The entire mixture was then diluted to 250 µl and 3 µl were spotted onto a PEI-cellulose strip which was subsequently developed in 0.27 M ammonium sulfate (Figure 7A) or in 0.8 M ammonium formate, pH 3.5 (Figure 7B).

2.2.2.6 Autoradiography and Quantitation

Autoradiography was carried out using Kodak XAR-5 and RP film. PEI-cellulose sheets used to assess normal nucleotides and excess ATP conditions were placed with film at 4° C for up to 30 minutes without enhancing screens. Two-dimensional adduct maps were autoradiographed using screen enhancement at -70° C for up to 72 hours. Following development of the film, detected adducts were excised from the chromatogram and counted by Cerenkov assay using a Beckman LS 7800 liquid scintillation counter. The relative adduct labelling index (RAL) was determined according to the following equation.

$$RAL = \frac{\text{c.p.m in adduct nucleotides}}{\text{c.p.m. in normal nucleotides x dilution factor}}$$

2.3 RESULTS

2.3.1 DNA adduct formation following 1,8-NONP treatment of E. coli

DNA adduct formation was examined after treatment of E. coli strain NR6113 with 1,8-NONP. Following isolation and digestion of the bacterial DNA, adducts were separated from the normal nucleotides and postlabelled using the butanol extraction procedure. The results are shown in Figure 8A. Three distinct spots corresponding to DNA adducts were detected. About 95 % of the adduct-related radioactivity was associated with spot #1. The two minor adducts (#2 and #3) each accounted for less than 2.5 % of the total DNA adduct formed in E. coli cells treated with 1,8-NONP. No adducts were detected in DNA obtained from E. coli treated only with DMSO (Figure 8B). The level of DNA modification following 1,8-NONP treatment was about 5 adducts per 10^7 nucleotides.

2.3.2 DNA adduct Formation by 1,8-NONP in vitro.

In order to obtain more information regarding the nature of the DNA lesions produced by 1,8-NONP, a number of experiments were carried out using adducts obtained from the reaction of 1,8-HANP with DNA in vitro. Previous studies in our laboratory showed that in vitro modification of calf thymus DNA (CT DNA) with 1,8-HANP at pH 5.0 yielded a single major DNA adduct which was identified as the guanine C(8) adduct, N-(2'-deoxyguanosin-8-yl)-1-amino-8-nitropyrene (dG-C(8)-ANP) (Andrews et al., 1986; Andrews, 1988). Analysis of 1,8-HANP modified CT DNA using either the standard ^{32}P-postlabelling technique (Figure 9A) or the butanol extraction procedure (Figure 9B) suggested that more adducts were present in the DNA than had been

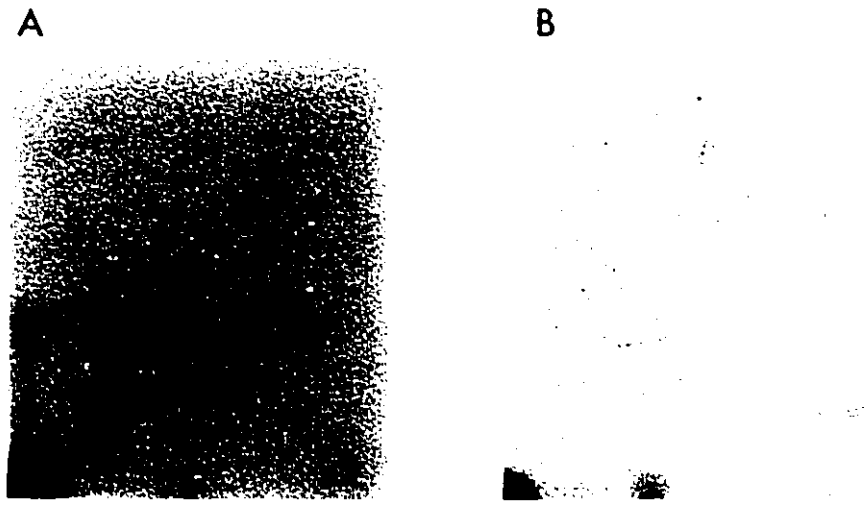


Figure 8. DNA adduct formation by 1,8-NONP in E. coli. Representative adduct pattern for ^{32}P-labelled 1,8-NONP adducts. Panel A. Fingerprint pattern of DNA adducts formed in E. coli following treatment with 1,8-NONP. Adducts were extracted with 1-butanol and labelled with excess carrier-free [γ-^{32}P]ATP. Adducts were resolved in D_3 and D_4 as described in the text. Panel B. Control experiment: ^{32}P-postlabelling of DNA obtained from E. coli treated with DMSO.

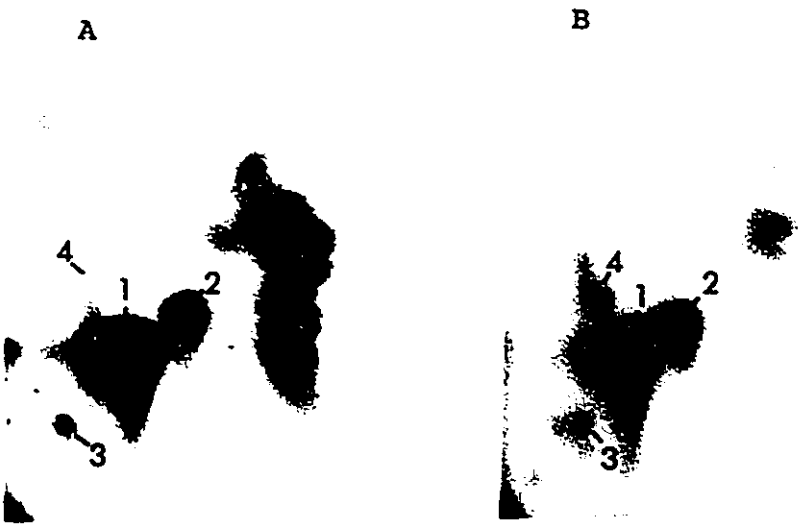


Figure 9. ^{32}P-postlabelling analysis of 1,8-HANP-modified calf thymus DNA.
Panel A: 1,8-HANP-modified CT DNA analyzed using the standard postlabelling protocol (Randerath et al., 1981; Gupta et al., 1982). Panel B: 1,8-HANP-modified CT DNA analyzed using the butanol extraction technique (Gupta, 1985). Chromatography conditions are described in the text.

detected in the experiments described by Andrews (1988). The fingerprint pattern of labelled DNA adducts (Figure 9A & B) contained a single major product (spot #1) and several minor products. The level of modification in these preparations was determined to be about one adduct per 10^3 nucleotides. No radioactivity appeared on the chromatogram when unmodified DNA was analyzed in a similar fashion. These results suggested that the new technique was either detecting unique adducts which were below the limits of detection in the previous studies, or that the experimental conditions were creating artifacts.

2.3.2.1 Products of Incomplete Digestion

In order to determine whether some of the detected products resulted from incomplete digestion of the DNA to 3'-phosphodeoxyribonucleosides, the digestion time was varied from 30 minutes to 4 hours. The results of this experiment are shown in Figure 10. An increase in the time of digestion of the 1,8-HANP modified CT DNA led to a marked diminution in the proportion of total radioactivity associated with the spots (#5-8) found in the upper right portion of the chromatogram. An increase in the percentage of the total radioactivity associated with spot #1 was also observed. This suggested that: 1) spots # 5-8 were products of incomplete digestion (adduct-oligonucleotides); and 2) adducted 3'-phosphodeoxyribonucleosides released from the oligonucleotide material by digestion might migrate to the same area on the chromatogram as spot #1. Similar experiments with untreated DNA did

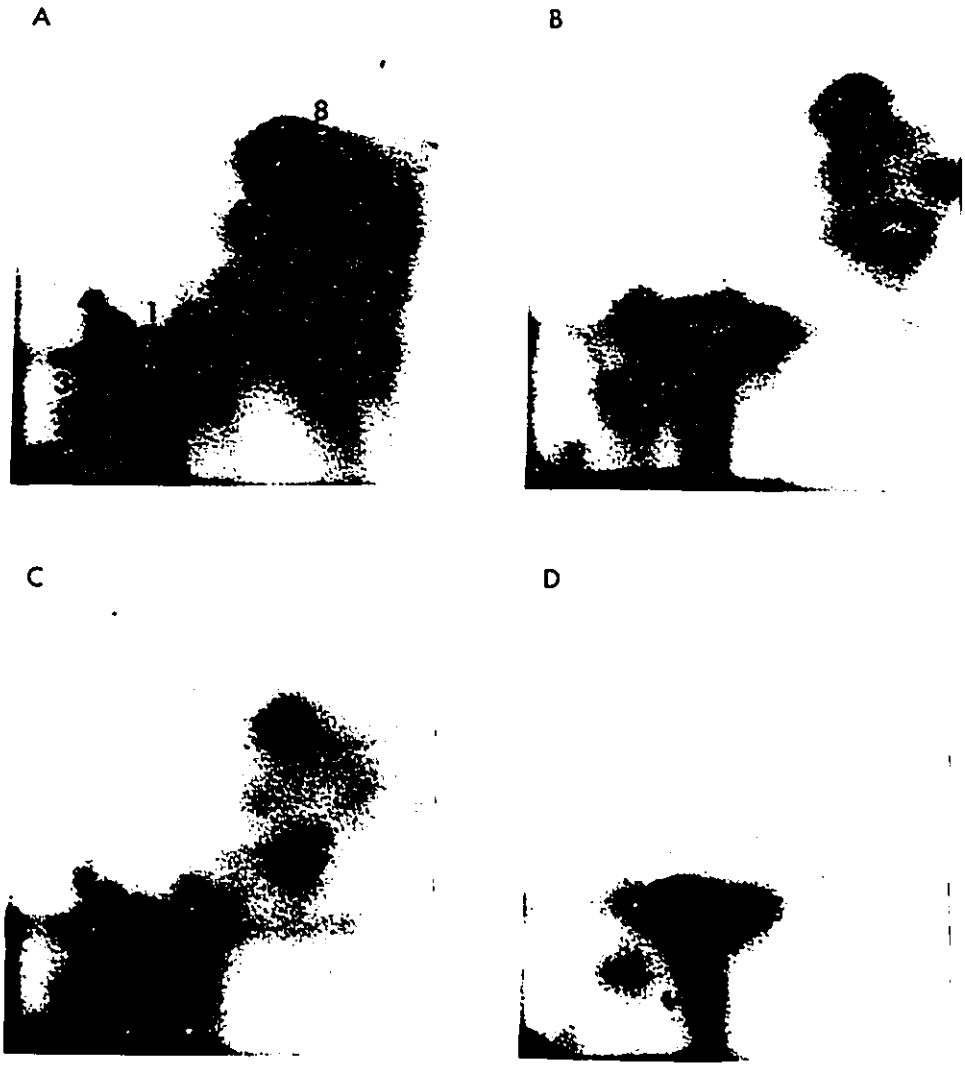


Figure 10. The effect of digestion on the ^{32}P-postlabelling pattern of 1,8-HANP-CT DNA adducts.
DNA was incubated with micrococcal nuclease and spleen phosphodiesterase. As the incubation progressed, 1.7 ul aliquots were removed and labelled with [γ-^{32}P]ATP using the standard postlabelling procedure (Randerath et al., 1981; Gupta et al., 1982). Digestion times were: Panel A 0.5 hour, Panel B 1 hour, Panel C 2 hours, Panel D 4 hours.

not show accumulations of radioactivity in the upper right region of the chromatogram, even at the shortest time of digestion.

2.3.2.2 Postlabelling of 1,8-NONP-treated Poly(dG.dC) DNA

To determine whether all the DNA adducts detected in CT DNA could be accounted for by the reaction of 1,8-NONP with dG:dC base pairs, DNA adducts were produced by reaction of 1,8-NONP with alternating poly(dG:dC) DNA. The results are shown in Figure 11. The pattern of 1,8-NONP adducts formed in poly(dG:dC) DNA (Panel B) was very similar to that formed in CT DNA (Panel A) suggesting that most of the adducts detected in CT DNA resulted from reaction of 1,8-HANP with either dG or dC. The major adduct (#1) formed in the poly(dG:dC) sample accounted for greater than 95 % of the adduct-related radioactivity. This adduct migrates to the same position on the TLC plate as the major adduct observed in digests of DNA obtained from E. coli treated with 1,8-NONP (Figure 8A).

A minor adduct which is consistently detected in CT DNA, and also in bacteria treated with 1,8-NONP (Figure 8A) is the lesion represented by spot #3. As shown in Figure 11B, this adduct is formed with either dG or dC. At least one other minor product is detected in poly(dG:dC) DNA, and migrates to the region of the chromatogram represented by spot #4. However, this adduct is not consistently detected in these experiments, and has not been detected in vivo (Figure 8A; Norman, 1988; Norman et al., 1989a).

2.3.2.3 Postlabelling of 1,8-NONP-treated Poly(dA.dT) DNA

The major difference between the DNA adducts formed in CT DNA (Figure 11A) and poly(dG:dC) DNA (Figure 11B) is the presence of spot

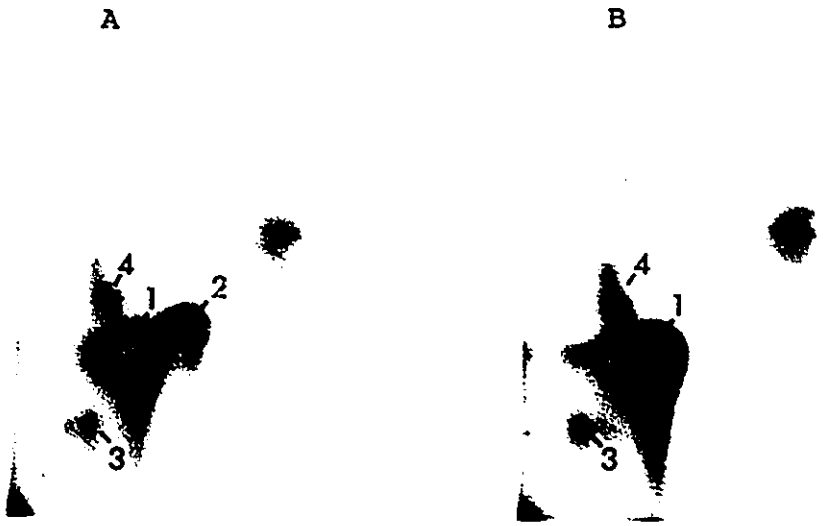


Figure 11. 1,8-HANP adducts in poly(dG.dC) DNA.
^{32}P-Postlabelling of 1,8-HANP adducts formed in CT DNA (Panel A) or poly(dG.dC) DNA (Panel B). The butanol extraction technique was used in these experiments. Chromatographic conditions are described in the text.

#2 in the CT DNA preparation. Different experiments done over several years have shown that, relative to spot # 1, the location of this spot on the chromatogram is not constant. For instance, in Figure 11A spot #2 runs further than spot #1 in the vertical direction (D_3), while in Figure 12A spot #2 does not run as far as spot #1 in D_3. Figures 11A and 12A are the results of different postlabelling experiments of the same 1,8-HANP-CT DNA preparation. Possible reasons for the variation in the location of this adduct will be considered in the Discussion. Since the lesion represented by spot #2 was not present in labelled digests of poly(dG:dC) DNA treated with 1,8-HANP (Figure 11B), it was considered likely that it represented an adduct of dA or dT. To test this possibility, 1,8-HANP was reacted with poly(dA:dT) DNA and subjected to postlabelling analysis. The result of this experiment is shown in Figure 12C. One major adduct and one minor adduct were detected. The major 1,8-HANP-poly(dA:dT) DNA adduct co-chromatographed with spot #2 in CT DNA (Figure 12A and 12B), suggesting that the adduct represented by spot #2 results from the reaction of 1,8-HANP with dA or dT. The similarity of the position of adduct #2 relative to adduct #1 observed in both CT DNA (Figure 12A) and E. coli (Figure 8A) suggests that the minor dA or dT adduct is also formed in E. coli.

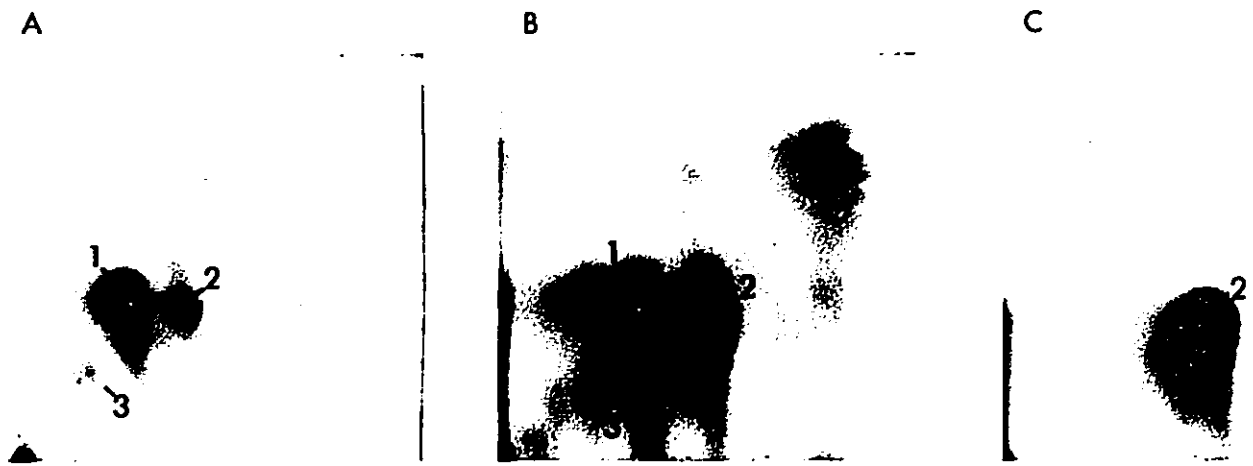


Figure 12. 1,8-HANP adducts in poly(dA.dT) DNA.
^{32}P-postlabelling of 1,8-HANP adducts formed in CT DNA (Panel A), CT DNA + poly(dA.dT) DNA (Panel B), and poly(dA.dT) DNA (Panel C). The butanol extraction procedure was used in these experiments.

2.4 DISCUSSION

2.4.1 The Major DNA Adduct Formed by 1,8-NONP in E. coli

The major adduct formed by 1,8-NONP in E. coli migrated to the same position on the TLC sheet as the major adduct formed in calf thymus DNA or poly(dG.dC) DNA which had been treated with 1,8-HANP at pH 5.0. Since it has been demonstrated by proton NMR, uv-visible, and fluorescence spectroscopy, and fast atom bombardment mass spectrometry that the major adduct formed by the in vitro reaction of 1,8-HANP with DNA is dG-C(8)-ANP (Andrews et al., 1986; Andrews, 1988), it is likely that the DNA adduct represented by spot #1 is the 3',5'-diphosphate derivative of dG-C(8)-ANP (Figure 13).

The dG-C(8)-ANP adduct is produced by the reaction of guanine with the arylnitrenium ion derived from 1,8-HANP. Therefore in bacteria treated with 1,8-NONP, adduct formation requires: 1) reduction of 1,8-NONP to 1,8-HANP; and 2) transformation of 1,8-HANP to the reactive arylnitrenium ion. The identity of the cellular factors in E. coli which metabolize 1,8-NONP to the DNA reactive species are not known. Reduction of 1,8-NONP to 1,8-HANP occurs as the result of a two electron transfer which might be catalyzed by any of the numerous nitroreductases which are known to be active in E. coli (McCalla, 1983). At physiological pH the direct reaction of 1,8-HANP with DNA is extremely slow (Andrews et al., 1986), presumably due to the slow rate of nonenzymatic transformation to the corresponding nitrenium ion. In S. typhimurium, and perhaps also in E. coli, formation of the ultimate mutagen requires the activity of acetyltransferase (Orr et al., 1985). The products of acetylation

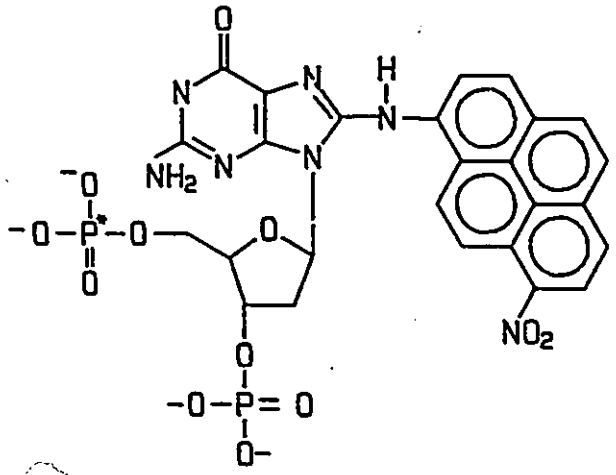


Figure 13. The major 1,8-NONP adduct.
This is the probable structure of the major DNA adduct (spot #1) formed by 1,8-HANP in CT DNA and poly(dG.dC) DNA, and by 1,8-NONP in E. coli. The structure shown is the 3', [^{32}P]5'-diphosphate of N-(2'-deoxyguanosin-8-yl)-1-amino-8-nitropyrne.

which have been isolated are N-acetylated derivatives; these are relatively stable, and only weakly mutagenic (Orr et al., 1985) and therefore they are unlikely to represent the ultimate mutagens. However, it is probable that the acetyltransferase also catalyses the formation of the O-acetyl derivative of 1,8-HANP, N-acetoxy-1-amino-8-nitropyrene (1,8-AANP) (Orr et al., 1985). Based on the chemical and biological properties of O-acetylated derivatives of aromatic amines it would be expected that 1,8-AANP would be highly unstable, and would undergo heterolytic cleavage to form the nitrenium/carbenium cation-acetate anion pair which would react with DNA (section 2.1.2; Kadlubar and Beland, 1985).

The observation that 1,8-NONP forms a major DNA adduct at the C(8) position of guanine is consistent with many previous studies which have demonstrated that most nitroarenes and aminoarenes (Figure 14) form DNA adducts primarily at dG-C(8) (Howard et al., 1983; Beland and Kadlubar, 1985; Andrews et al., 1986; Gupta and Early, 1988). Moreover, the ability of several N-substituted aryl compounds to induce mutations in bacteria and CHO cells (Beland et al., 1983; Howard et al., 1983; Heflich et al., 1986b) and sister chromatid exchanges (SCEs) in CHO cells (Heflich et al., 1986b) has been correlated with the degree of modification at the C(8) position of guanine.

2.4.2 Minor DNA Adducts formed with 1,8-NONP in E. coli

At least two minor adducts (#2 and #3) are formed by 1,8-NONP in E. coli. These adducts are also formed in CT DNA following treatment with 1,8-HANP. The experiments described here provide the

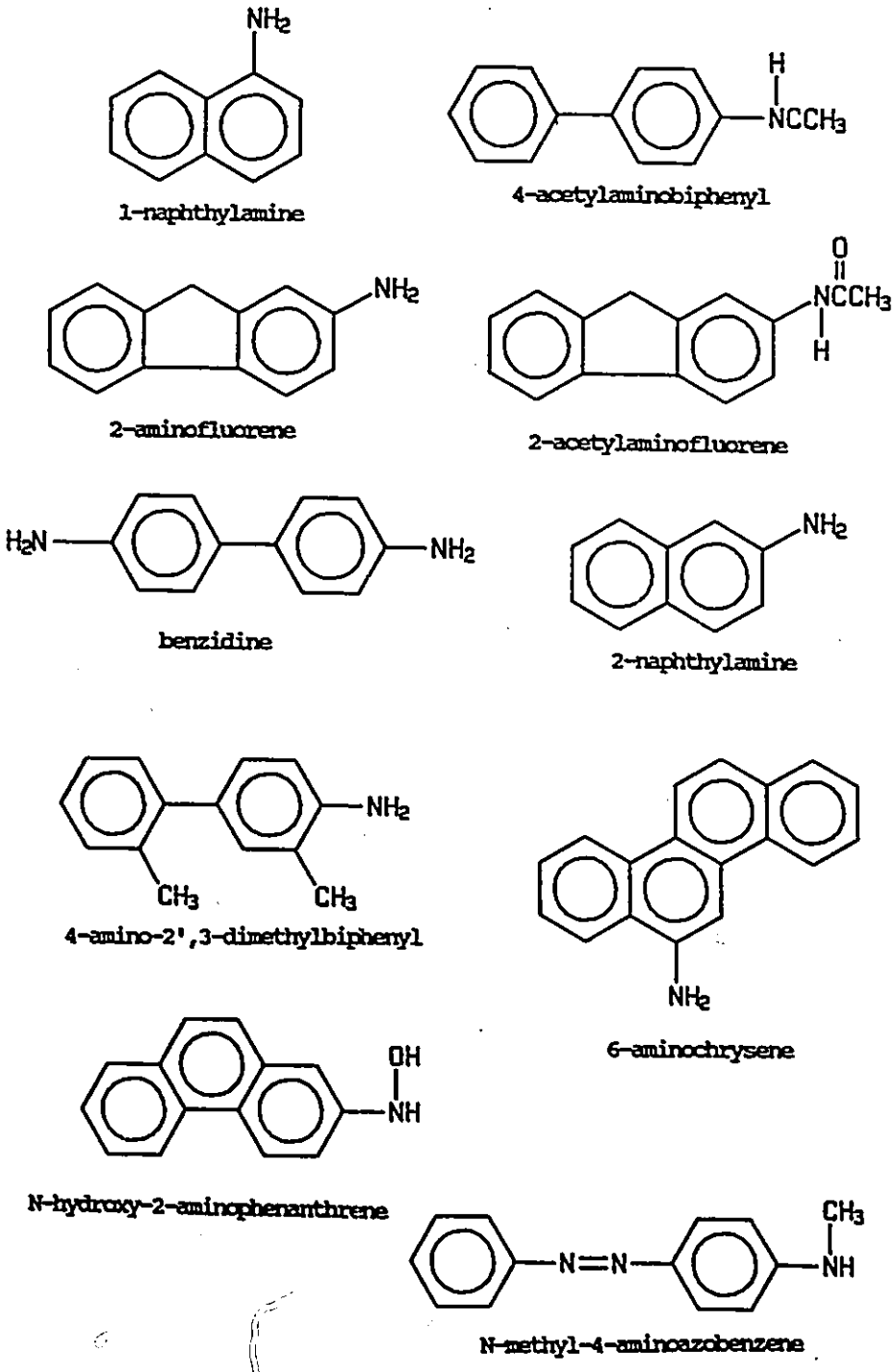


Figure 14. The structures of several aromatic amine derivatives.

first evidence that dinitropyrene derivatives can form DNA adducts other than dG-C(8)-ANP.

2.4.2.1 Adduct Formation With dA or dT

Adduct #2 is the major DNA adduct formed in poly (dA.dT) DNA with 1,8-HANP. In E. coli, an adduct is formed with 1,8-NONP which migrates to the same position on the TLC plate as the major 1,8-HANP-poly(dA.dT) adduct. These observations suggest that spot #2 in E. coli is a product of the reaction of the nitrenium/carbenium ion derived from 1,8-NONP with either dA or dT. In the in vitro system, an additional minor adduct is formed in poly(dA.dT) which is not present in either E. coli treated with 1,8-NONP, or CT DNA treated with 1,8-HANP.

At least 3 different types of DNA adducts resulting from the reaction of N-hydroxy arylamines with dA have been reported in the literature. Adducts formed (via an ortho carbenium cation) at the N^6 position of dA were characterized in the liver and urothelium of dogs treated with 2-napthylamine (Kadlubar et al., 1981), in the liver of rats treated with N-methyl-4-aminoazobenzene (Tullis et al., 1981), and following in vitro treatment of calf thymus DNA with either N-hydroxy-2-napthylamine (Kadlubar et al., 1980), or N-acetoxy-2-acetylaminophenanthrene (Gupta and Early, 1988). Adducts formed at the C(8) position of dA following reaction of a nitrenium ion were detected in the urothelium of dogs treated with 4-aminobiphenyl (Beland et al., 1983), and are also formed in vitro by reaction of either N-hydroxy-4-aminobiphenyl or N-hydroxy-3,2'-dimethyl-4-aminobiphenyl with CT DNA (Beland et al., 1983; Flamming et al.,

1985). In addition, reaction of N-acetoxy-2-acetylaminofluorene with either poly(dA.dT) (Harvin et al., 1977) or polyadenylic acid (Kriek and Rietsema, 1971) resulted in adducts which were modified at the C(8) position of adenine. Finally, a novel C(8)-modified derivative of deoxyinosine (dI) was characterized in the lung and livers of preweanling mice treated with 6-nitrochrysene (6-NC) or 6-aminochrysene (6-AC) (Delclos et al, 1987a), in rat hepatocytes treated with 6-NC or 6-AC (Delclos et al., 1987b), and in CT DNA treated in vitro with N-hydroxy-6-aminochrysene (Delclos et al., 1987b). The dI-C(8) adduct is believed to be a product of oxidation of the corresponding dA-C(8) adduct (Delclos et al., 1987b).

The structures of hypothetical DNA adducts which might result from the interaction of 1,8-HANP with dA are shown in Figure 15. These structures are based on analogous dA adducts formed by several other N-hydroxyarylamines as discussed above.

There have been no reports of adducts formed with dT by N-hydroxy arylamines. On the basis of quantum mechanical calculations, McCoy et al. (1985b) have proposed that O^4 and O^2 would be the most probable sites for DNA adduct formation on dT; however, the reactivity of these positions towards nitrenium/carbenium electrophiles is much lower than that of the N(3), N^6, N(7), or C(8) positions of dA.

The position of adduct #2 on the TLC plate varied relative to the major DNA adduct: early determinations of the postlabelling pattern of 1,8-HANP-CT DNA adducts (Figure 11A) showed that the minor adduct migrated further in the vertical direction than adduct #1,

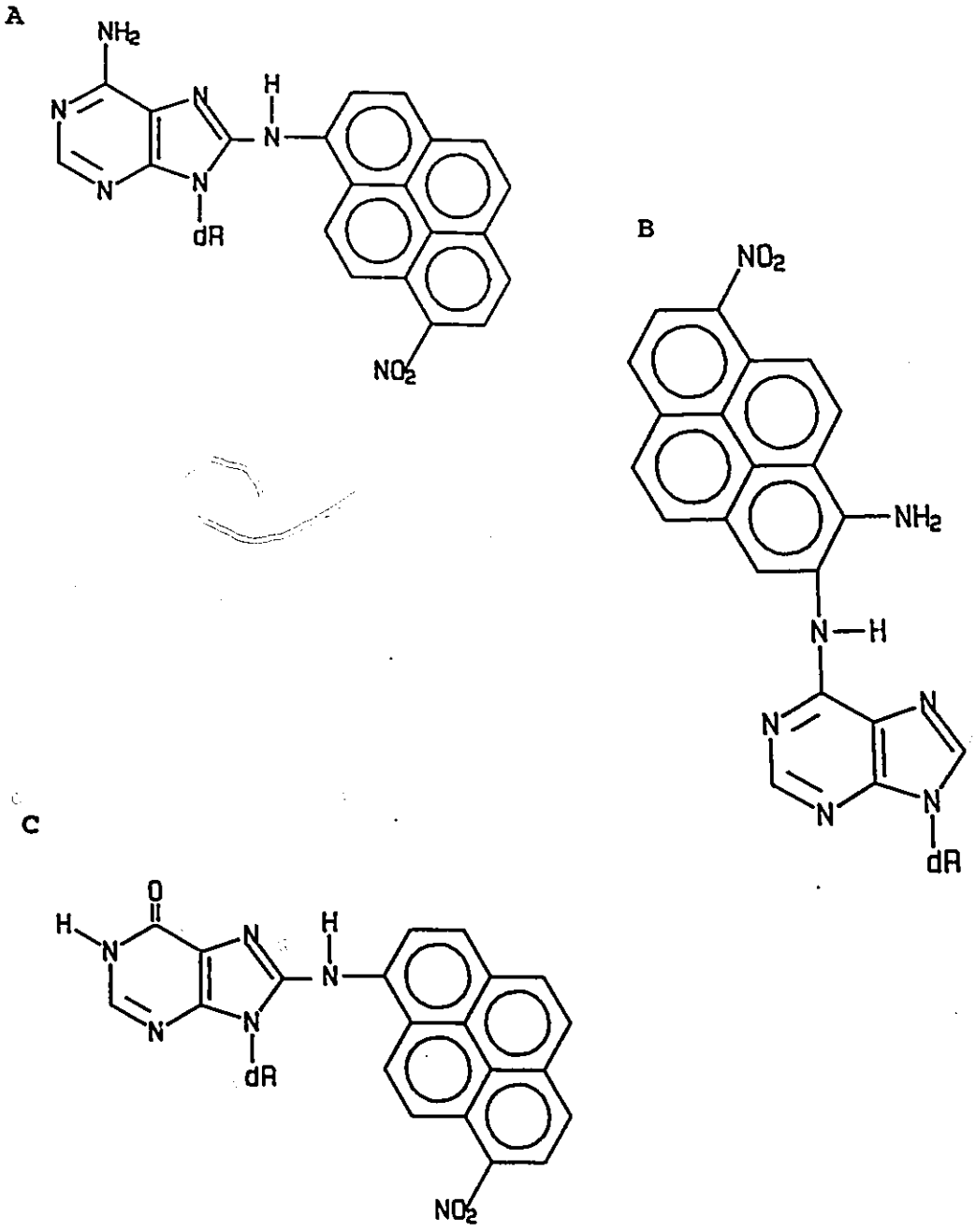


Figure 15. Possible structures of the adduct represented by spot #2. A) N-(2′-deoxyadenosin-8-yl)-1-amino-8-nitropyrene. B) 2-(2′-deoxyadenosin-N^6-yl)-1-amino-8-nitropyrene. C) N-(2′-deoxyinosin-8-yl)-1-amino-8-nitropyrene. The adduct on the TLC would be the 3′, [^{32}P]5′-diphosphate of the adduct-nucleoside shown. These structures are based on analogous adducts which are formed by aromatic amine compounds, as described in the text.

while later experiments using the same DNA sample (Figure 12A) showed that adduct #2 did not migrate as far as adduct #1. There are at least two possible explanations for the anomalous chromatographic behaviour of this adduct. 1) The adduct might have broken down to a product with different chromatographic properties as the result of oxidation or imidazole ring opening. 2) If the adduct were formed with adenine, then the chromatographic behaviour might be explained by differential protonation of the adenine in solvents of slightly different pH. Since a 3',5'-diphosphodeoxynucleoside derivative of an adenine adduct would be expected to have a pK for purine ring protonation of between 3.5 and 4, it is possible that the spot that migrates further in the vertical direction than adduct #1 represents the protonated form of a 3',5'-diphosphodeoxyadenosine adduct, while the spot with lower mobility represents the unprotonated form of the same adduct. In this regard it is notable that the distance in which the adduct(s) represented by spot #2 migrate on the TLC vary only in the vertical dimension (D_3: 7 M urea, 2.5 M ammonium formate, pH 3.5); they migrate the same distance in the horizontal direction (D_4: 7 M urea, 0.8 M LiCl, 0.5 M Tris-HCl, pH 8.0). Because of the high ionic strength of the solution and the inclusion of high concentrations of urea it is possible that different preparations of the D_3 solution had slightly different pH's. The major guanine adduct would not be influenced by differences in pH of either solution since the pKs for guanine protonation/deprotonation would be less than 2.5 and greater than 9.

2.4.2.2 Minor Adducts of dG or dC

Adduct #3 is a minor adduct formed in E. coli treated with 1,8-NONP, and in CT DNA or poly(dG.dC) DNA following treatment with 1,8-HANP at pH 5.0. This adduct could represent either a breakdown product of dG-C(8)-ANP or a unique adduct formed with dG or dC. Other than the C(8) position, the most common positions on dG for adduct formation with N-hydroxy arylamines are the exocyclic N^2 and O^6 positions. Adducts resulting from reaction of an ortho carbenium ion with the N^2 position of dG were detected in the urothelium of dogs treated with 2-napthylamine (Kadlubar et al., 1981), the livers of rats and mice treated with 4-acetylaminobiphenyl (Gupta and Dighe, 1984), N-methyl-4-aminoazobenzene (Beland et al., 1980) or N,N-dimethyl-4-aminoazobenzene (Delclos et al., 1984), and several tissues of rodents treated with 2-acetylaminofluorene (AAF) (reviewed in Beland and Kadlubar, 1985). Adducts were also characterized at the N^2 position of dG following in vitro treatment of calf thymus DNA with N-hydroxy-2-napthylamine (Kadlubar et al., 1980), N-hydroxy-4-aminobiphenyl (Beland et al., 1983), and N-hydroxy-2-aminophenanthrene (Gupta and Early, 1988). N-hydroxy-1-napthylamine forms adducts at the O^6 position of dG in vitro (Kadlubar et al., 1978), and also in rats (Dooley et al., 1984). The linkage between the aryl moiety and dG-O^6 can be through either the arylamine nitrogen or the ortho carbon (Kadlubar et al., 1978). It should be noted however that N-hydroxy-1-napthylamine is the only N-substituted aryl compound which is known to form dG-O^6 substituted products (Beland et al., 1983). Figure 16 shows the structures of minor

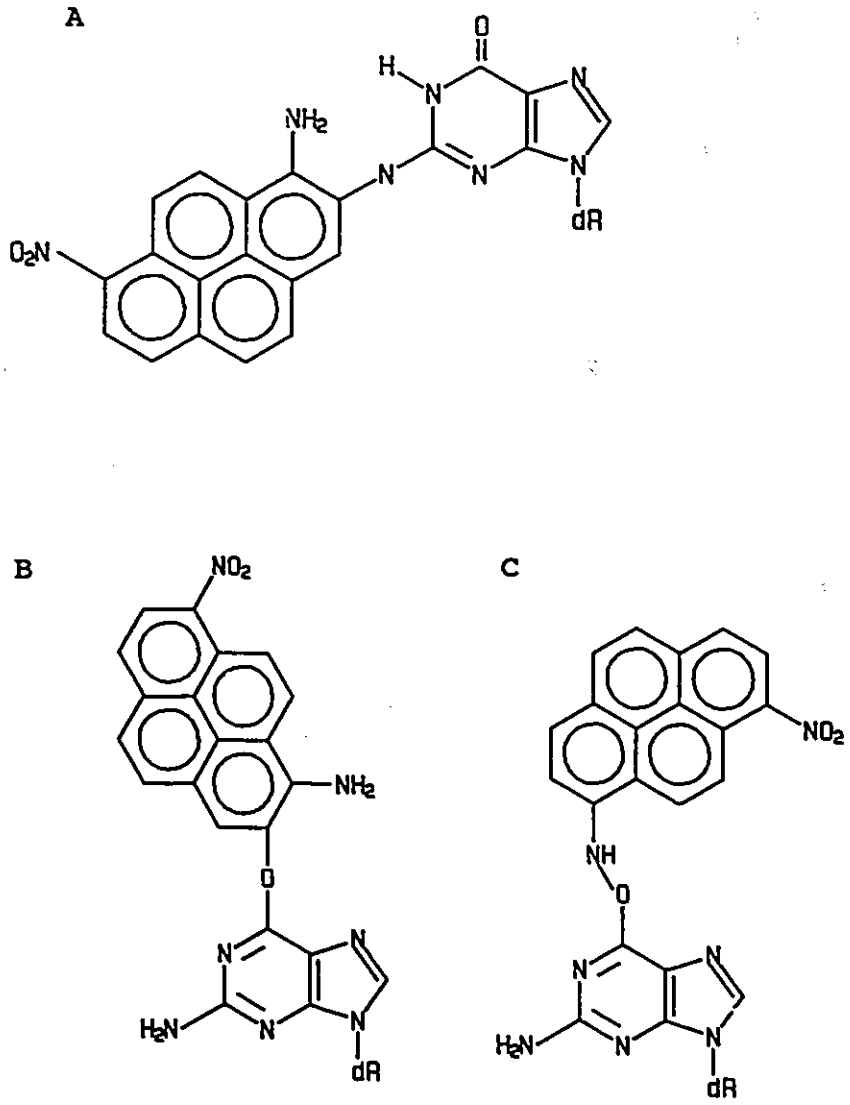


Figure 16. Possible structures of the adduct represented by spot #3. A) 2-(2'-deoxyguanosin-N^2-yl)-1-amino-8-nitropyrene. B) 2-(2'-deoxyguanosin-O^6-yl)-1-amino-8-nitropyrene. C) N-(2'-deoxyguanosin-O^6-yl)-1-amino-8-nitropyrene. The adduct on the TLC would be the 3', [^{32}P]5'-diphosphate of the adduct-nucleoside shown. These structures are based on analogous adducts which are formed by aromatic amine compounds, as described in the text. It is also possible that the adduct represented by spot # 3 is a breakdown product of the major adduct shown in Figure 13.

adducts which might result from the reaction of 1,8-HANP with dG. Spot #3 could be the 3',[^{32}P]5'-diphosphonucleoside of one of these lesions.

2.4.2.3 Products of Incomplete Digestion

Postlabelling analysis of DNA which had been modified in vitro with 1,8-HANP detected four diffuse spots which migrated to the upper right portion of the chromatogram. The observation that increased digestion resulted in a decrease in the amount of radiolabel associated with this material suggests that it is the result of incomplete digestion of the modified DNA. Gupta (1984; 1985) has shown that DNA modified with AAF also yields four adducts which exhibit very similar chromatographic behaviour. The AAF adducts were identified as adduct dinucleotides of the structure dpXpNp, where X is the dG-C(8) adduct of AAF and N is A, T, C, G (Gupta, 1984). It is possible that the 4 spots detected in 1,8-HANP-modified DNA are analogous adduct dinucleotides formed with dG-C(8)-ANP. The adduct might physically interact with the nucleases, or alter the conformation of the oligonucleotide such that digestion at sites immediately adjacent to the adduct is decreased.

2.4.3 ^{32}P-Postlabelling of 1,8-NONP Adducts: Advantages and Limitations

The experiments described in this chapter show that the ^{32}P-postlabelling technique is an extremely sensitive method for detecting DNA adducts formed by 1,8-NONP. Evidence has been presented that, in addition to the major dG-C(8)-ANP adduct, 1,8-NONP also forms minor adducts. This is significant as there have

been no previous reports in the literature of minor adducts formed by dinitropyrenes, or partially reduced derivatives of dinitropyrenes. The possible identity of the minor lesions was discussed in section 2.4.2. In E. coli the minor adducts were present at a level of about 1 adduct per 10^8 nucleotides. Other studies in our laboratory (Norman, 1988; Norman et al., 1989a) have shown that these relatively low adduct levels are still about 2 orders of magnitude above the detection limit when the butanol extraction technique is used. Moreover, the level of DNA modification in a relatively small DNA sample (1-5 μg) can be determined. The ability to detect and quantitate low levels of DNA adducts formed by 1,8-DNP and 1,8-NONP have made it possible to conduct other studies in our laboratory which have determined the rates of DNA adduct formation and removal in mammalian cells in culture, and in rodents (Norman, 1988; Norman et al., 1989a; see section 4.2).

A significant disadvantage of the ^{32}P-postlabelling technique is that the technique does not provide structural information about detected lesions. In the present study this problem has been partially overcome by using an in vitro activation system. This has provided some evidence as to the possible identities of the detected lesions. Nevertheless, unambiguous characterization of all these products will require the synthesis of adducted 3', [^{32}P]5'-diphosphonucleoside standards and studies of the chromatographic behaviour of these products.

3. THE MUTATIONAL SPECIFICITY OF 1,8-NONP: INFERENCES FOR MUTATIONAL MECHANISM

3.1 INTRODUCTION

Many mutagenic agents form bulky DNA adducts. These include polyaromatic hydrocarbons such as benzo(a)pyrene (BP), aromatic amines such as 2-acetylaminofluorene (AAF), mycotoxins such as aflatoxin B_1 (AFB_1), and nitroaromatic compounds such as 1,8-DNP. In bacteria, the mutagenicity of these agents is in large part dependent on cellular functions encoded by the SOS regulon. In the past 15 years considerable progress has been made in understanding the regulation of the SOS response in bacteria, and the biological role of many of the gene products in mutagenesis (Witkin, 1976; Walker, 1984; Peterson et al., 1988). Additional information regarding mutational mechanism can be obtained by determination of mutational specificity which refers to both the types of mutations which are induced (mutagenic specificity), and the sites at which they occur (site specificity) (Miller, 1983). Such studies can provide insight into the role of various modulating factors on the mutational process including: 1) the effect of DNA repair enzymes; 2) the nature of DNA sequences adjacent to mutational sites; and 3) the identity of premutagenic lesions (Miller, 1983). This chapter provides a detailed description of the mutational specificity of 1,8-NONP in the lacI gene of E. coli.

The following sections provide background information

relevant to the experimental approach used in these studies, and the analysis of the results. First, the lacI mutagenesis system in E. coli will be reviewed. Second, DNA repair enzymes will be reviewed with particular emphasis on the components of the inducible error-prone repair pathway and nucleotide excision repair.

3.1.1 Determination of the Mutational Specificity of Mutagens

The specificity of mutation has been of longstanding interest to scientists. Early studies by Freese and coworkers showed that mutagens such as hydroxylamine, proflavine, or the base analogues 2-aminopurine and 5-bromo-deoxyuridine had distinctive mutagenic specificities in phage T4 (Freese, 1959; Freese et al., 1961). These observations were central to the formulation of early mutational mechanisms (Freese, 1959; Brenner et al., 1961), and were also important for the elucidation of the genetic code (Crick et al., 1961). At about the same time, Benzer (1961) showed that mutation did not occur at random within a gene, but rather at specific "hotspots". In addition, different mutagenic agents induced mutations at distinct sites within the rII region of phage T4 (Benzer, 1961). These early observations demonstrated that mutagens exhibit both site specificity and mutagenic specificity. In subsequent years, determination of the specificity of mutation has provided valuable insight into the process of mutation (for reviews see Drake, 1970; Drake and Baltz, 1976; Miller, 1983; and Eisenstadt, 1987).

Sophisticated systems for the study of mutational specificity have been developed which make it possible to determine the precise

DNA sequence of induced mutations (Eisenstadt, 1987). Reversion systems have frequently been used because of their rapidity, high sensitivity, and the perception that the results provide accurate information regarding mutagenic specificity (Eisenstadt, 1987). The most commonly used assays of this type are reversion of trpA alleles in E. coli (Yanofsky, 1971), and of his alleles in S. typhimurium (Maron and Ames, 1983). The disadvantage of these assays is that they monitor mutation at relatively few sites. In addition, recent evidence suggests that the types of mutational events which cause reversion to amino acid prototrophy are not as restricted as was once believed (Hartman et al., 1986). Therefore, further analysis of the revertants (i.e. Miller and Barnes, 1986) is required in order to assess the mutational specificity accurately.

Several different forward mutation systems have been used to gather information regarding the specificity of mutagens in bacteria (reviewed in Eisenstadt, 1987, and references therein). Forward mutation systems are generally more informative because of the large number of sites which can be monitored simultaneously. However, a considerable amount of work is required to determine mutagenic specificity. A general strategy which has been quite successful is to select mutations which occur on extrachromosomal targets such as episomes, small plasmids, and phages. Such mutations are amenable to genetic and DNA sequencing analysis since they can be easily transferred into strains of diverse genetic background for mapping and cloning purposes, and for the determination of suppression patterns (Eisenstadt, 1987). In addition, the mutated genes which

are resident on plasmid and phage elements can be directly isolated for further molecular analysis.

Analysis of the results obtained from forward mutation systems requires knowledge of the mutational target. For instance, most frameshift mutations, large deletions and additions, and nonsense mutations will eliminate gene function to such an extent that the resultant mutants have a markedly altered phenotype. On the other hand, missense mutations will yield a variety of phenotypes, only some of which will be sufficiently different from the wild-type to alter growth on selective media. Thus, the most useful information regarding mutational specificity will be obtained from a system whose mutational target has been studied extensively; this allows estimation of the types of mutations which can be detected and the sites at which they occur.

3.1.1.1 The lacI system of E. coli

Use of the lacI gene of E. coli for mutational studies in bacteria was pioneered by Jeffrey Miller (reviewed in Miller, 1978; 1983). A shuttle vector containing the lacI gene has since been developed for use in mammalian cells (Lebkowski et al., 1985; DuBridge et al., 1987). The following discussion will deal only with the bacterial system.

The lacI gene in E. coli encodes the lactose repressor. Early studies by Francois Jacob and Jacques Monod (reviewed in Jacob and Monod, 1961) identified the repressor as a regulatory element which binds reversibly to the lactose operator to control the activity of the structural genes encoding β-galactosidase, lactose

permease, and transacetylase. These workers showed that in the presence of an inducer such as isopropyl-β-D-thiogalactoside (IPTG), the repressor was inactivated and the cellular levels of β-galactosidase were increased by up to 10,000-fold relative to the uninduced state. High levels of β-galactosidase were also observed in the absence of an inducer in mutants containing a defective repressor (lacI$^-$ mutations). In 1966, Gilbert and Muller-Hill purified the lactose repressor using radioactive IPTG as a marker. They found that the repressor was a tetrameric protein with identical 38 kDa subunits (Gilbert and Muller-Hill, 1966). Subsequent DNA sequence analysis of the lacI gene showed that each subunit consisted of 360 amino acids encoded by a 1080 base pair gene (Farabaugh, 1978). In vitro, the repressor was shown to bind to DNA containing the lactose operator; dissociation of the repressor-operator complex occurred in the presence of IPTG (Gilbert and Muller-Hill, 1967). Extensive mutational analysis of the repressor showed that it contained several functional domains (reviewed in Muller-Hill, 1975; Miller, 1978; Gordon et al., 1988). The initial 60 amino acids comprise the DNA binding domain while the remaining 300 amino acids constitute the protein core which mediates inducer binding, subunit aggregation, and transmission of the induction signal from the inducer-binding site to the DNA binding domain (Muller-Hill, 1975; Miller, 1978).

In 1977 Miller and his colleagues published several papers which outlined the basic elements of a genetic system, using the lacI gene, which could be applied to the study of mutational specificity

(Miller et al., 1977; Schmeissner et al., 1977a; 1977b; Coulondre and Miller, 1977a; 1977b). First, a fine structure deletion map of the lacI gene was constructed which consisted of over 100 intervals with an average spacing of about 10 nucleotides per interval. This was done using E. coli strains in which a derivative of phage ϕ80 carrying the lac operon had been integrated into the chromosome near the tonB locus (Beckwith and Signer, 1966). A large number of deletion mutants extending from the tonB locus into the lacI gene were collected. The order within the lacI gene, and approximate physical endpoints, of these deletions was determined by crossing them against point mutations of known identity (Schmeissner et al., 1977a). The resultant fine structure deletion map provided a means by which the location of any new mutation within the lacI gene could be readily determined by a series of recombination assays (deletion mapping). The next step in development of this system was accomplished by analyzing the suppression patterns of over 5000 nonsense mutations within an episomal lacI gene using the tRNA suppressor species Su1, Su2, Su3, Su6, SuB, SuC, and Su5 (Miller et al., 1977; Coulondre and Miller, 1977a). The location of these nonsense mutations within the lacI gene was also determined by deletion mapping. By comparing the suppression patterns and location of the nonsense mutations to the protein sequence of the lactose repressor (Beyreuther, 1973), the majority of these mutations were assigned to a location in the gene corresponding to a specific residue of the repressor. The results showed that out of 90 codons which could yield nonsense mutations, 65 base substitution events

would give rise to an amber or ochre mutation via a single base change at an independent site (Coulondre and Miller, 1977b; Coulondre et al., 1978; Miller et al., 1978). Following determination of the DNA sequence of the lacI gene (Farabaugh, 1978), it was possible to verify the assignments which had been made strictly on the basis of the protein sequence (Miller et al., 1978).

The development of this system has made it possible to monitor nonsense mutations accurately and rapidly solely by genetic means. LacI$^-$ mutants can be selected by plating cultures on phenyl-β-D-galactoside (PGal). Since the mutant lacI gene is resident on an episomal factor, it can be conjugally transferred into appropriate bacterial strains to facilitate either deletion mapping or suppression analysis. The results of this analysis allow amber and ochre mutations to be attributed to specific transition and transversion events. This system has several advantages: 1) the mutational target is well defined both with respect to mutagenic specificity and site specificity; 2) since only nonsense mutations are monitored, selection bias does not alter the mutational spectrum; and 3) all possible base substitutions except A:T => G:C transitions can be monitored.

An obvious disadvantage of this system is that it is only applicable to nonsense mutations. In order to analyze other mutational events, Calos (1978) constructed the pMC1 and pMC4 derivatives of plasmid pMB9. LacI$^-$ mutations can be cloned into these plasmids by in vivo recombination (Calos and Miller, 1981). Recombinants containing the lacI$^-$ mutation can be amplified, a

restriction fragment containing the mutation isolated, and the DNA sequence determined by Maxam and Gilbert sequencing. This strategy has been used to examine the DNA sequence of frameshift mutations (Calos and Miller, 1981; Miller, 1985), deletions (Farabaugh et al., 1978; Albertini et al., 1982), and missense mutations (Miller, 1985). Two features of this cloning/sequencing strategy are disadvantageous: 1) the frequency of recombination between the episome containing the mutation ($\underline{I}^-\underline{Z}^+$) and the plasmid ($\underline{I}^+\underline{Z}^+$) is often of the same order of magnitude as the frequency of $\underline{lacI}^-$ formation on the plasmid, making it difficult to clearly ascertain the origin of the mutation; and 2) the relatively laborious process of isolating restriction fragments precludes rapid analysis of large collections of mutations.

To circumvent the above problems, Schaaper et al. (1985) developed a procedure which facilitates the efficient recovery of $\underline{lacI}^-$ mutations from a F'lac onto a single-stranded M13 vector, mRS81 (Figure 17). This vector contains the entire lacI gene, the lactose operator, and the gene segment encoding the α-complementation region of lacZ. A single point mutation in the lacZ segment has eliminated α-complementation activity. Consequently, the genotype of mRS81 is $\underline{lacI}^+\underline{Z}^-\alpha$ (Schaaper et al., 1985). Recovery of the $\underline{lacI}^-$ mutation onto the cloning vector is based on homologous recombination between the episome ($\underline{lacI}^-\underline{Z}^+\alpha$) and the M13 phage. Only recombination involving the simultaneous transfer of the adjacent lacI and lacZα genes from the F'lac to the phage will yield a $\underline{lacI}^-\underline{Z}^+\alpha$ M13 vector. $\underline{LacI}^-\underline{Z}^+\alpha$ recombinants can be easily identified as blue plaques in an α-complementation host which is $\underline{LacI}^-\underline{Z}^-\alpha$. Following isolation and

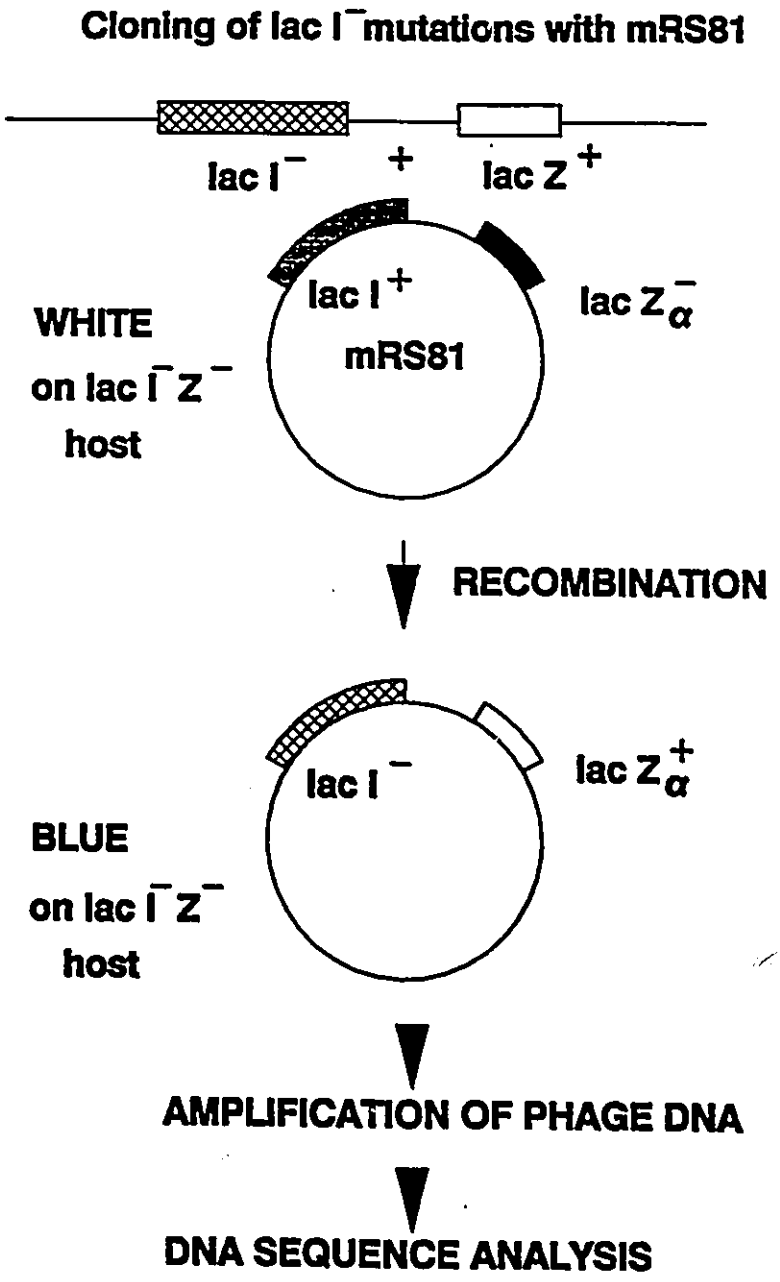


Figure 17. Cloning of lacI⁻ mutations into mRS81.
F'lacI⁻ mutations are recovered in the M13 vector mRS81 following in vivo recombination. Recombinant lacI⁻Z⁺α phage form blue plaques in an α-complementation host which is lacI⁻Z⁻α (Schaaper et al., 1985).

purification of the recombinant phage, large amounts of single stranded viral DNA can be prepared for subsequent DNA sequence analysis using the dideoxy chain termination method (Sanger et al., 1980). This system contains several advantages over methods which use pMB9 derivatives (Calos, 1978): 1) the requirement for a double change from lacI$^+$Z$^-\alpha$ to lacI$^-$Z$^+\alpha$ reduces the possibility that the lacI$^-$ mutation which is recovered is the result of spontaneous mutation occurring on the phage; 2) the mutation of interest is recovered onto a single stranded vector which can be sequenced directly; and 3) by dispensing with the requirement for the isolation of restriction fragments, large numbers of mutants can be handled simultaneously.

Selection of lacI$^-$ mutants is much easier when mutation studies are done with lac operons which contain promoter mutations. The lacI gene has an unusually weak wild-type promoter which functions constitutively (Calos, 1978). Consequently, selection of lacI$^-$ mutations on PGal is frequently inefficient; in order to avoid growth of i$^+$ colonies, selective media must be pretreated with scavenger strains to remove alternate carbon sources from the agar (Smith and Sadler, 1971; Miller, 1972). Even when these precautions are taken, a large proportion of the colonies which form on PGal are lacO^c mutants (Smith and Sadler, 1971). In order to increase the efficiency of lacI$^-$ selection, the episomal lac operon which is used in studies of mutational specificity contains two promoter mutations. The first is the I^q mutation which results in a ten-fold increase in the synthesis of lactose repressor (Muller-Hill et al., 1968). The

second is the L8 mutation in the lac promoter (Scaife and Beckwith, 1967) which causes a 17-fold decrease in the expression of lac structural proteins (Reznikoff and Abelson, 1978). The combination of these two mutations results in extremely tight regulation of the lac enzymes by the lactose repressor, allowing clean selection of lacI mutants on PGal medium without the use of scavenger strains (Miller et al., 1977; Miller, 1978). When lac operons containing the I^qL8 mutations are used for mutagenesis, most of the colonies which form on PGal are expected to be lacI$^-$; operator-constitutive (O^c) mutants are rare when the I^q and L8 promoters are used (Miller, 1978).

The techniques described above have been used in the present study. This is illustrated in Figure 18 and described in greater detail in Methods (Section 3.2.2). Mutant lacI$^-$ genes selected following mutagenic treatment were resident on an F' episomal factor which could be readily transferred into a variety of different genetic backgrounds in order to determine such parameters as nonsense suppressibility (Miller et al., 1977; Coulondre and Miller, 1977a), approximate location of the mutation in the gene (by deletion mapping) (Schmeissner et al., 1977a), and dominance in the presence of a wild type repressor (Miller, 1972). Information from the deletion mapping and dominance experiments was used to choose an appropriate DNA sequencing strategy. The lacI$^-$ genes were cloned into the M13 phage mRS81 (Schaaper et al., 1985), and the DNA sequence determined using the dideoxy chain termination technique (Sanger et al., 1980).

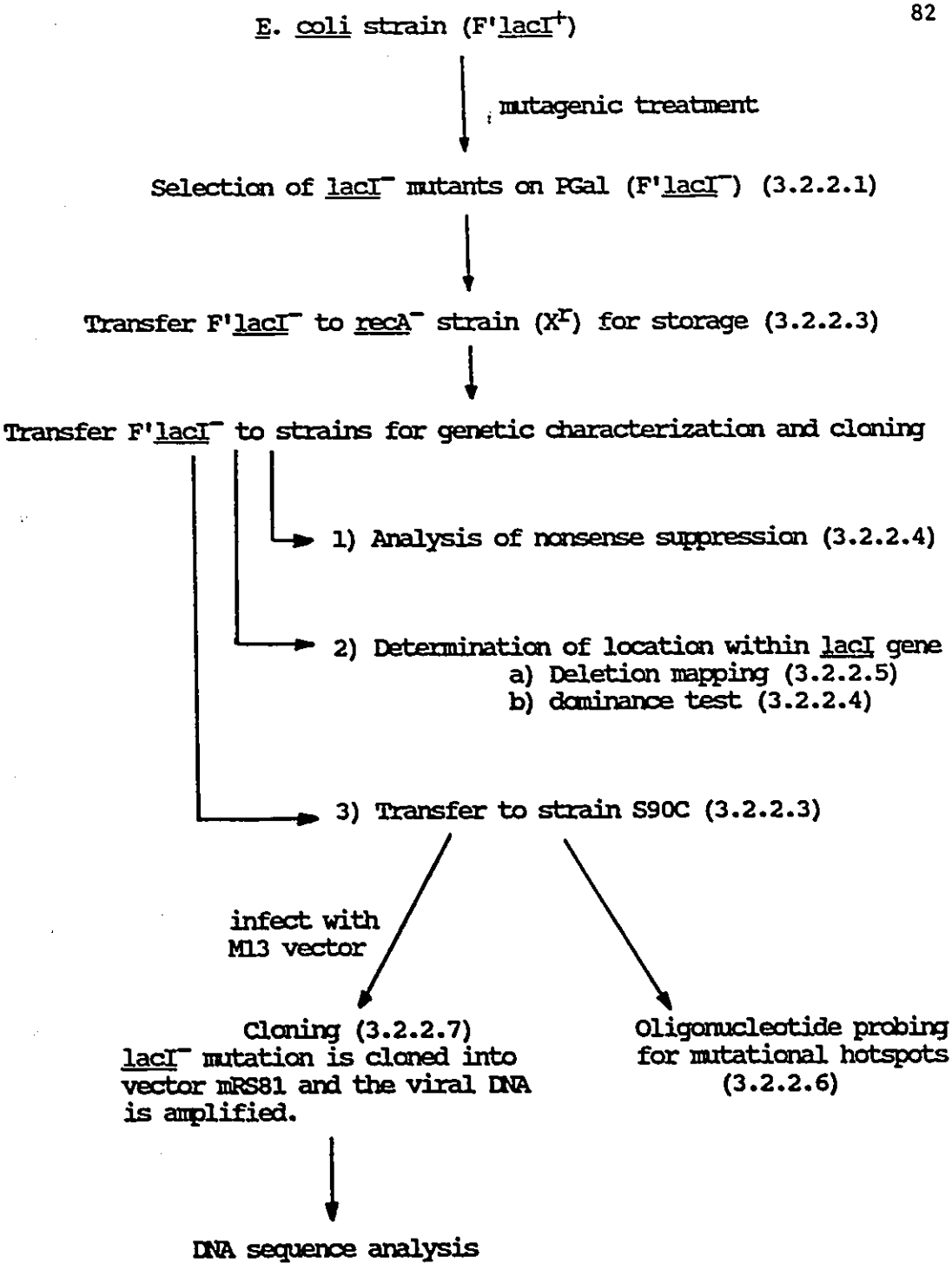


Figure 18. Strategy for characterization of lacI- mutations. The number in parentheses denotes the section in Methods where the procedure is described in detail.

3.1.2 DNA Repair

A variety of different DNA repair pathways allow cells to tolerate DNA damage induced by mutagens. The major modes of repair include excision repair (Sancar and Sancar, 1988; Friedberg, 1985); mismatch repair (Modrich et al. 1987; Radman, 1988); recombinational repair (Walker, 1985; 1987; Sancar and Sancar, 1988); and damage reversal through photoreactivation (Sutherland, 1981) or adaptive methyl transfer (Lindahl and Sedgwick, 1988). Many DNA repair enzymes are members of 3 regulatory networks which are induced by DNA damage: the SOS regulon (Walker, 1984; 1987), the adaptive response (Lindahl and Sedgwick, 1988), and the oxidative damage response (Linn and Imlay, 1987). In addition to inducible error-free DNA repair pathways, E. coli also contain inducible mutagenesis functions which constitute a form of DNA repair which is intrinsically error-prone. The DNA repair literature is enormous, and even a cursory review of different pathways is beyond the scope of the present discussion. Error-prone repair and nucleotide excision repair are relevant to the work reported here. Therefore the following discussion will be restricted to these topics.

3.1.2.1 Error-prone Repair

The SOS regulatory system was first described following the observation that a number of different phenomena such as viral reactivation, prophage induction, filamentous growth, and mutagenesis could be induced in E. coli by DNA damaging agents (reviewed in Witkin, 1976; Walker, 1984). A model which could account for the genetic dependence of the SOS functions was proposed by Gudas and

Pardee (1975). These authors suggested that a number of inducible functions were negatively regulated by a repressor (encoded by the lexA gene) which could be inactivated by the RecA protein. Subsequent studies showed that RecA protein facilitated proteolytic cleavage of the LexA repressor (Little et al., 1980). Cleavage of the LexA repressor results in derepression of approximately 20 unlinked operons (Ossanna et al., 1987) including those whose products are involved in excision repair, recombination, cell division, and mutagenesis (Walker, 1984).

The genes required for SOS mutagenesis in E. coli include recA, umuC, and umuD (Kato and Shinoura, 1977; Steinborn, 1978). The inducible gene products appear to be required to permit DNA synthesis past lesions which would otherwise block replication. However, this synthesis is "error-prone" and results in increased levels of mutagenesis. E. coli mutants which do not contain the mutagenesis functions are nonmutable by UV light or several chemical mutagens, and are also more sensitive to the lethal effects of such agents (Kato and Shinoura, 1977; Steinborn, 1978).

The umuC and umuD genes have been cloned and sequenced (Elledge and Walker, 1983; Shinagawa et al., 1983; Perry et al., 1985). The genes are organized in an operon which is repressed by LexA protein. The UmuD protein exhibits considerable homology to LexA repressor at the putative RecA protease cleavage site (Perry et al., 1985). Recently it has been shown that UmuD is cleaved by a reaction which requires RecA (Burkhardt et al., 1988; Shinagawa et al., 1988). It has been proposed that the role of RecA in the cleavage of UmuD is

to accelerate the tendency of UmuD to undergo self-proteolysis (Burkhardt et al., 1988). Cleavage of UmuD is necessary for SOS mutagenesis (Nohmi et al., 1988; Shinagawa et al., 1988). It has been shown that the carboxyl-terminal fragment is sufficient for the role of UmuD in mutagenesis (Nohmi et al., 1988). Although the precise function of the umuCD gene products in mutagenesis is unknown, it is believed that these proteins are required for the resumption of processive DNA replication following incorporation of nucleotides opposite a bulky DNA adduct (Bridges and Woodgate, 1985).

The plasmid pKM101 is one of many which affect survival and mutagenesis in bacterial cells treated with SOS-dependent mutagens (reviewed in Strike and Lodwick, 1987). This plasmid contains the mucA and mucB genes whose products are the functional homologs of the bacterial UmuD and UmuC proteins, respectively. The mucAB operon shares 52% homology with the umuCD operon at the nucleotide level (Perry et al., 1985). The UmuD homolog, MucA, has a cleavage site at virtually the same location as UmuD; mucB encodes a protein which is homologous to UmuC (Perry et al., 1985). The introduction of plasmid pKM101 restores SOS mutagenesis capability to umu$^-$ mutants; mutagenesis mediated by pKM101 is dependent on a recA$^+$ lexA$^+$ genotype in the host bacterium (Walker and Dobson, 1979; Walker, 1984). Plasmid pKM101 can also enhance the SOS response in umu+ cells (Mattern et al., 1985). This might be due to increased gene dosage: the plasmid pKM101 is present in bacteria at approximately 7 copies per cell (Levin et al., 1982). In addition, MucA might be activated more easily than UmuD as the result of efficient posttranslational

modification by autoproteolysis in the absence of activated RecA protein (Shinagawa et al., 1988).

The variety of phenotypes exhibited by different recA mutants in E. coli (reviewed in Little and Mount, 1982; Walker, 1984; Cox and Lehman, 1987; Smith, 1988; Dutreix et al., 1989) suggest that the RecA protein is required for genetic recombination, prophage induction and SOS mutagenesis. In mutagenesis, RecA protein has at least 2 well documented functions which have been discussed above: 1) to initiate induction of the SOS regulon by cleaving the LexA repressor (Little et al., 1980); and 2) to activate the UmuD protein posttranslationally (Nohmi et al., 1988; Shinagawa et al., 1988; Burkhardt et al., 1988). In addition, it is believed that RecA protein has a third, as yet incompletely defined, function in SOS mutagenesis. This might involve inhibition of the 3' => 5' proofreading activity of DNA polymerase, or a reduction in the fidelity of base insertion (Fersht and Knill-Jones, 1983; Lu et al., 1986; Lu and Echols, 1987; Jonczyk et al., 1988; Dutreix et al., 1989).

A considerable amount of evidence suggests that E. coli DNA polymerase III (PolIII) is required for SOS mutagenesis (Bridges et al., 1976; Hagansee et al., 1987). PolIII can incorporate nucleotides opposite the site of a bulky lesion or a noncoding apurinic site and continue replication, but only with difficulty (Livneh, 1986; Hevroni and Livneh, 1988; Shavitt and Livneh, 1989). Factors which could inhibit processivity include: 1) the proofreading activity encoded by the subunit ϵ, which might excise

newly incorporated bases causing the polymerase to undergo repeated incorporation-excision cycles at the site of the lesion (Lu et al., 1986; Lu and Echols, 1987); or 2) interaction of the lesion with the processivity factor β of PolIII, causing the polymerase to dissociate from the DNA prior to, or following, incorporation of a base opposite the lesion (Shavitt and Livneh, 1989). In this regard, it is possible that UmuC/D facilitates processive replication by clamping the polymerase to DNA. This would decrease the probability that the polymerase will dissociate from the DNA (Hevroni and Livneh, 1988). PolIII synthesis is induced slightly (2-fold) during SOS induction (Bonner et al., 1988). An SOS-inducible DNA polymerase (PolX) has been characterized which is believed to be DNA polymerase II (PolII) (Bonner et al., 1988). This polymerase is capable of incorporating nucleotides opposite noncoding lesions (abasic sites) and then continuing DNA synthesis. Elucidation of the precise role, if any, of this polymerase in SOS mutagenesis requires further investigation.

3.1.2.2 Excision Repair Mediated by the UvrABC Eximuclease Complex

Nucleotide excision repair involves the removal of an oligonucleotide containing DNA adducts, followed by DNA repair synthesis to fill in the resultant gap. A number of proteins facilitate this process, including UvrA, UvrB, UvrC, UvrD (helicase II), PolI, and DNA ligase (Sancar and Sancar, 1988). The genes for four of these products (uvrA, uvrB, uvrC, and uvrD) are regulated by the LexA repressor and can be transcriptionally activated following induction of the SOS regulon (Peterson et al., 1988).

The experimental evidence gathered to date (for review see Freidberg, 1985; Freidberg, 1987; Sancar and Sancar, 1988; Myles and Sancar, 1989) have led to the development of the following model of nucleotide excision repair. 1) The UvrA protein dimerizes in the presence of ATP, and binds to a single subunit of UvrB. 2) The UvrAB complex binds to DNA at the site of a DNA adduct. 3) UvrC binds to the UvrAB-DNA ternary complex and hydrolyzes the 8th phosphodiester bond 5' from the DNA adduct, and the 5th phosphodiester bond 3' from the adduct. 4) DNA helicase II and PolI displace the uvrABC complex and the excised oligomer, and fill in the gap using the undamaged strand as a template. 5) DNA ligase seals the gap at the 3'-end of the nascent oligomer. The resultant repair patches are predicted to be 12-13 nucleotides long.

Nucleotide excision repair is considered to be essentially error-free (Walker, 1984). Therefore mutagenesis by agents which form bulky DNA adducts is expected to be low in an excision repair proficient (uvr$^+$) host. However, mutagenesis might occur in uvr$^+$ strains if: the DNA adduct is not efficiently recognized by the UvrAB complex (Burns et al., 1986; 1988a); the level of DNA damage overwhelms the capacity of the repair enzymes to remove the adducts; DNA replication occurs prior to excision repair; or PolI encounters a second lesion in the template strand during gap filling (Witkin, 1976). In contrast, mutagenesis in a uvr$^-$ strain would be expected to occur primarily during semi-conservative DNA replication, or postreplicatively during DNA synthesis within daughter strand gaps (Schaaper et al., 1987).

3.1.3 Experimental Approach

The purpose of the experiments described in this chapter was to obtain a detailed description of the mutational specificity of 1,8-NONP in the *lacI* gene of *E. coli*. Following treatment with 1,8-NONP, *lacI*$^-$ mutants were selected from four strains in *E. coli* with different DNA repair capabilities: strain NR6113 was deficient in excision repair due to a deletion through the *uvrB* locus; EE125 contained the plasmid pKM101; CM6114 was both deficient in excision repair capability and contained the plasmid pKM101; NR6112 contained a wild-type DNA repair background.

3.2 MATERIALS AND METHODS

3.2.1 Materials

3.2.1.1 Chemicals

L-Tryptophan, L-methionine, thiamine, and streptomycin were purchased from Gibco/BRL. Biotin, ampicillin, DL-dithiothreitol (DTT), salmon sperm DNA, sephadex G-50, nalidixic acid, and rifampicin were obtained from Sigma Chemical Co.. Bacto agar, yeast extract, and bactotryptone were obtained from Difco. Glucose, NaCl, Na_2HPO_4, Na_2EDTA, $MgCl_2.6H_2O$, Na_2 tetraborate, polyethylene glycol-8000 (PEG), DMSO, phenol, Na acetate, and formamide were purchased from BDH Chemicals. Chloroform was purchased from Caledon Laboratories Ltd.. NEN-colony/plaque screen NEF-978 nylon filters, and 5'-[γ-^{32}P]ATP (>3000 Ci/mmol) were purchased from NEN-Dupont. Deoxyadenosine 5'-[α-^{35}S]thiotriphosphate triethylammonium salt ([α-^{35}S]dATPαS) (>1000 Ci/mmol) was purchased from Amersham. Tris, SDS, bromophenol blue, xylene cyanol, acrylamide, bis-acrylamide, TEMED, ammonium persulfate, and urea were obtained from Biorad Laboratories. DNA polymerase I (Klenow fragment: 5000 U/ml), polynucleotide kinase (EC 2.7.1.78), dideoxynucleoside triphosphates (ddNTPs), and deoxynucleoside triphosphates (dNTPs) were purchased from Pharmacia (Canada) Inc.. PGal (phenyl-β-D-galactoside), XGal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), and TONPG (O-nitro-phenyl-β-D-thiogalactoside) were purchased from Research Organics Inc. (Cleveland, Ohio). All oligonucleotide probes and primers were purchased from the Central Facility of the Institute for Molecular Biology and Biotechnology (McMaster University, Hamilton). The

synthesis of 1,8-NONP (purity > 99%) by oxidation of 1-amino-8-nitropyrene was previously described by Andrews et al. (1986).

3.2.1.2 Bacteria and Bacteriophage

All bacterial strains were derivatives of E. coli K12. Their properties and sources are given in Table 5. A number of lacI$^-$ controls were used for oligonucleotide probing studies and deletion mapping. These are listed in Table 6. The F'lac used in these studies carries the I^Q (Muller-Hill et al., 1968) and I8 (Scaife and Beckwith, 1967) promotor mutations. The bacteriophage M13mRS81 was obtained from B.W. Glickman, York University, Toronto. The genotype of this phage is M13lacI^{Q}Z$^-$αT129,I8 (Schaaper et al., 1985).

3.2.1.3 Media

The composition of the media used in these studies is described in Miller (1972). Amino acids and vitamins, when required, were added to media at the following concentrations: tryptophan 50 mg/l, methionine 50 mg/l, biotin 500 µg/l. All media except Luria-Bertani (LB) broth were supplemented with thiamine (5 mg/l). Unless otherwise stated, the agar concentration on plates was 1.6 % (w/v). Rich (LB) medium contained bactotryptone (10 g/l), yeast extract (5 g/l), and NaCl (10 g/l). Minimal plates contained Vogel-Bonner (VB) salts (Vogel and Bonner, 1956) and glucose (0.2 %). PGal minimal plates contained PGal (phenyl-β-D-galactoside) (75 mg/l) instead of glucose as a carbon source. X-Gal plates were minimal plates supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (40 mg/l). TONPG plates contained VB salts, succinate (0.33 %), and TONPG (O-nitrophenyl-β-D-thiogalactoside) (300 mg/l).

Table 5. Bacterial strains used for selection and characterization of *lacI*$^-$ mutations.

Strain	Sex	Genetic Markers	Reference or Source
NR9099	F'(*lacI*$^-$ *pro*$^+$ *lacZ*$^-$ M15)	*ara* Δ(*lac pro*) *thi* *recA56*	Schaaper *et al.*, 1985
CSH52	F$^-$	*ara* Δ(*lac pro*) *thi* *strA* (φ80d*lac*) *recA*	Miller, 1972
S90C	F$^-$	*ara* Δ(*lac pro*) *thi* *strA*	Miller *et al.*, 1977
X^r	F$^-$	*ara* Δ(*lac pro*) *thi* *recA*	B.W. Glickman
EE125	F'*lac pro*	*ara* Δ(*lac pro*) *thi* Tsv^r(CV^s) pKM101	Gordon *et al.*, 1988a
NR6112	F'*lac pro*	*ara* Δ(*lac pro*) *thi* Tsv^r(CV^s)	B. W. Glickman
NR6113	F'*lac pro*	*ara* Δ(*lac pro*) *thi* Tsv^r(CV^s) Δ(*uvrB bioFCD chlA*)	B. W. Glickman
CM6114	F'*lac pro*	*ara* Δ(*lac pro*) *thi* Tsv^r(CV^s) Δ(*uvrB bioFCD chlA*) pKM101	Gordon *et al.*, 1988a
NR3951	F' *lac pro*	*ara* Δ(*lac pro*) *thi* *trpE9777* Δ(*bioFCD-uvrB-chlA*)	Schaaper *et al.*, 1987
XA93	F$^-$	*ara* Δ(*lac pro*) *thi* *nalA* *argE*-am *rif* su3	Miller *et al.*, 1977
XA9B	F$^-$	same as XA93 except suB	Miller *et al.*, 1977
XA9C	F$^-$	same as XA93 except suC	Miller *et al.*, 1977
DF1131 DF1116 DF1032 DF1142 DF1050 DF1033	F$^-$	*ara* Δ(*lac pro*) *galE* *strA* (φ80d*lac*) *thi* pAA31, with *tonB* deletions extending into the *lacI* gene on the φ80d*lac*	Gordon *et al.*, 1988a

All strains are derivatives of *E. coli* K12.

Table 6. Controls for genetic and probing studies.

Mutant #	Sequence Change	Use
1-91	+5'-TGGC-3' at 621-632	+ve control for +TGGC oligonucleotide probe
2-76	-5'-TGGC-3' at 621-632	+ve control for -TGGC oligonucleotide probe
1-17	C(90) => T	+ve control for wild type oligonucleotide probe +ve control for negative complementation in CSH52
1-51	G(631) => A	-ve control for all oligonucleotide probes
2-20	G(31) => A	+ve control for 1st deletion mapping interval -ve control for suppression strains
1-98	C(260) => T (ochre11)	+ve control for 1st deletion mapping zone -ve control for negative complementation in CSH52 +ve control for ochre suppression
3-28	C(377) => T (ochre17)	+ve control for 2nd deletion mapping zone +ve control for ochre suppression
1-13	G(381) => A	+ve control for 2nd deletion mapping zone -ve control for suppression strains
1-46	C(569) => T (ochre21)	+ve control for 3rd deletion mapping zone +ve control for ochre suppression
1-57	C(653) => T (ochre24)	+ve control for 4th deletion mapping zone +ve control for ochre suppression
1-22	C(770) => T (amber26)	+ve control for 5th deletion mapping zone +ve control for amber suppression
1-29	C(953) => T (amber33)	+ve control for 6th deletion mapping zone +ve control for amber suppression

All strains were obtained from B. W. Glickman. The mutations are resident on an episomal F'lac, and have been described in Gordon et al. (1988b).

PGal/Glucose/Ampicillin (PGal/Glu/Amp) plates contained VB salts, PGal (500 mg/l), glucose (0.1 %), and ampicillin (50 mg/l). When added to minimal medium streptomycin (Strp) was present at 200 mg/l, nalidixic acid (Nal) 40 mg/l, and rifampicin (Rif) 100 mg/l. XGal top agar contained VB salts, 0.8 % (w/v) agar, and 40 mg/ml XGal.

3.2.2 METHODS

3.2.2.1 Selection of lacI⁻ Mutants

Multiple cultures of CM6114, NR6113, EE125, and NR6112 were grown overnight at 37° C in a shaking waterbath. Ampicillin (50 µg/ml) was added to CM6114 and EE125 to maintain plasmid pKM101. Overnight cultures were pelleted by centrifugation, and resuspended in VB. Four 1 ml aliquots of each culture were transferred to 1.5 ml Eppendorf tubes. One hundred µl of DMSO was added to one of the tubes (control); 3 tubes were treated with 25 nmoles 1,8-NONP in 100 µl DMSO. After a 15 minute incubation at 37° C the bacteria were pelleted by centrifugation. The pellet was then washed and the bacteria were resuspended in VB and immediately placed on ice. Mutant (lacI⁻) frequency and survival were determined by plating appropriate dilutions of the cultures onto PGal and LB plates, respectively. PGal is a substrate for β-galactosidase but is not an inducer of the enzyme. Therefore when PGal is present as the sole carbon source, only cells which constitutively express β-galactosidase (ie. which carry lacI⁻ or lacO^C mutations) form colonies (Smith and Sadler, 1971). PGal plates were incubated for 2 days at 37° C, and LB plates for 16 hours. Mutation frequencies for both control (spontaneous) and 1,8-NONP-treated cultures were

calculated as mutants per 10^6 survivors. Eight lacI$^-$ colonies were isolated from each independently treated culture. The combination of short treatment period (15 minutes) in buffer, and direct plating onto PGal immediately following treatment (no cell division prior to plating) ensures that all induced mutants are of independent origin (See Section 3.3.1; Schaaper et al., 1987; Burns et al., 1988a).

Spontaneous mutants were selected in a slightly different manner. Multiple saturated cultures of NR6112, NR3951 (uvrB$^-$), EE125, or CM6114 were diluted in LB to a concentration of about 1000 cells/ml. Aliquots (200 µl containing about 200 cells) of each diluted culture were distributed into single wells of a 96 well microtitre dish. After overnight growth at 37^o C, the contents of each well was diluted in VB medium, and plated onto LB medium (to determine cell number) or PGal medium (to select lacI$^-$ mutants). One mutant was selected from each PGal plate to ensure independence (Schaaper et al., 1986).

3.2.2.2 Replica Matings

The lacI gene used in these studies is resident on an episomal factor which can be transferred from strain to strain by F' mediated conjugation. Transfers of this nature were facilitated by replica mating techniques (Miller, 1972). Mutants were picked to a specific position on a grid which was located on a master plate (50 to 100 mutants/plate) the night before matings were conducted. In the morning, the master grid was replicated onto fresh LB plates, grown for 6 hours, and then replicated onto a bacterial lawn derived by spreading 0.2 ml of a freshly saturated culture of recipient

strain on a selective plate (Miller et al., 1977). The selective plates were incubated at 37° C overnight and the colonies were then replicated onto an appropriate indicator plate. Recipient strains were delta(lacpro) and therefore proline auxotrophs. Donor strains were sensitive to a variety of antibiotics, or were methionine auxotrophs. Sexductants containing the F'lacpro could therefore be selected for concomitant proline prototrophy and antibiotic resistance, or for concomitant proline and methionine prototrophy.

3.2.2.3 Transfer to Storage Strain X^r

Following selection, mutants were picked onto a PGal master plate, and mated with Su3. The selection was concomitant Rif resistance and proline prototrophy. Sexductants were picked, regridded on Rif plates, and mated with the recA⁻ storage strain X^r. Selection was concomitant methionine and proline prototrophy. Sexductants were picked, and streaked to give single colonies on minimal medium plates. A single colony derived from each mutant was transferred to: 1) storage tubes containing LB broth; and 2) a master grid on minimal plates. The storage tube was incubated overnight at 37° C, and then stored at -70° C. DMSO (50 µl) was added to each tube as a cryoprotectant. Mutants gridded onto minimal plates were replicated into strain S90C. Selection was Strp resistance and proline prototrophy. S90C sexductants were replicated onto XGal indicator plates to confirm the i⁻ phenotype. Mutants which were white in S90C, or which transferred their episomal factor poorly were discarded. All F'lacI⁻ mutations used as controls for probing, suppression, dominance, or deletion mapping studies (Table

6) were also stored in X^r.

3.2.2.4 Dominance and Suppression Analysis

Most mutations which are in the portion of the lacI gene which encodes the DNA-binding domain of the lac repressor confer the I^{-d} (dominant) phenotype (Muller-Hill, 1975, Miller, 1978) and can be identified using a complementation assay (Miller, 1972). X^r strains (lacI$^-$ mutants and lacI^{-d} controls) were gridded onto minimal medium and mated with CSH52 which contains a wild-type repressor and is RecA$^-$. Selection was for streptomycin resistance and proline prototrophy. Sexductants were replicated onto XGal plates and incubated for 12 hrs at 37^o C. Mutant repressors encoded by the F'lacI$^-$, which were dominant to the wild-type repressor encoded by the chromosomal CSH52 lacI, exhibited negative complementation (ie. the lacI^{-d} phenotype) and were identified by the blue colour of the CSH52 sexductants on XGal medium (Miller, 1972).

The suppressibility of nonsense mutations was determined in strains carrying tRNA suppressors (Su3 (amber), SuB and SuC (ochre)) (Miller et al., 1977; Coulondre and Miller, 1977a). X^r strains (lacI$^-$ mutants and suppression controls) were gridded onto minimal plates and mated with suppressor strains. Selection was concomitant rifampicin resistance and proline prototrophy. Sexductants were replicated onto XGal plates containing nalidixic acid (XGal/Nal). Suppressed mutations were white on XGal/Nal medium.

3.2.2.5 Deletion Mapping

The approximate location of the mutation in the lacI gene was determined by deletion mapping using the galE$^-$ selection described by

Schmeissner et al. (1977a). X^r strains (lacI$^-$ mutants and mapping controls) were gridded onto minimal plates and mated with strains of the DF1000 series harboring the deletions 131, 116, 32, 142, 50, and 33 (Schmeissner et al., 1977b) which contain nonlethal tonB deletions extending into the 5' end of the lacI gene (Schmeissner et al., 1977a). The endpoints of these deletions are at positions 300, 410, 570, 695, 805 and 980 of the lacI gene (Schmeissner et al., 1977b). LacI$^+$ recombinates were amplified in two steps, first by replication onto TONPG medium and then onto PGal/Glu/Amp plates. TONPG is toxic to cells expressing lactose permease (ie. lacI$^-$ cells) when succinate is used as a carbon source. Galactose (the product of B-galactosidase cleavage of PGal) is toxic to galE$^-$galK$^+$ (galactose epimerase$^-$/ galactokinase$^+$) cells (Davies and Jacob, 1968) and therefore PGal selects against lacI$^-$ cells in this background. LacI$^+$ cells utilize the glucose as a carbon source and will not cleave PGal. Growth following the PGal/Glu/Amp selection was scored as lacI$^+$. Results from the deletion mapping studies and the dominance test were used to select the appropriate primer to begin DNA sequencing.

3.2.2.6 Oligonucleotide Probing

Sixty to 70 percent of spontaneous lacI$^-$ mutations result from the addition or deletion of the sequence 5'TGGC-3'from the sequence 5'-TGGCTGGCTGGC-3' at position 621-632 (Farabaugh et al., 1978; Schaaper et al., 1986). These mutations were detected by probing with oligonucleotides complementary to the wild-type or mutant sequences (wild-type probe: 5'-TATGCCAGCCAGCCAGACGC-3'; +TGGC

probe: 5'-TATGCCAGCCAGCCAGCCAGACGC-3'; -TGGC probe: 5'-TTTATGCCAGCCAGACGCAG-3') as described by Miller and Barnes (1986). X^r strains (*lacI*$^-$ mutants and probing controls) were mated with S90C as described above (section 3.2.2.3). S90C sexductants were gridded directly onto NEN colony/plaque screen NEF-978 nylon filters on minimal plates and grown overnight at 37° C. Mutant DNA was fixed by placing the filter on Whatman 3MM filter paper soaked with 0.5 N NaOH. The filters were washed, first with 0.5 M Tris-HCl (twice) and then 2X SSC (twice), air-dried and placed in a petri dish containing 4 ml preprobing solution (0.9 M NaCl, 0.05 M Na_2HPO_4, 5 mM EDTA, 1 % SDS, 250 µg/ml salmon sperm DNA) per filter, and rotated gently at room temperature for 1 hour. Twenty picomoles of the appropriate probe was incubated with 20 µCi [γ-^{32}P]ATP, 1.5 µl 10X kinase buffer (0.5 M Tris-HCl pH 7.6, 0.1 M $MgCl_2$, 50 mM DTT) and 4 units of T4 polynucleotide kinase (PNK) in a 15 µl reaction volume for 20 minutes at 37° C. The mixture was then diluted to 100 µl and the labelled probe was purified on Sephadex G-50 using the spun column procedure (Maniatis *et al.*, 1982). One hundred µl of the purified labelled probe was added to the petri dish containing the filters, the dish sealed with parafilm, placed in a lucite container and rotated at 37° C overnight. The following morning, filters were washed twice with 6X SSC for 10 minutes at room temperature, twice for 10 minutes at 37° C, and finally for 10 minutes at the appropriate restrictive temperature. Filters were air-dried at room temperature and autoradiographed overnight using Kodak XAR-5 or RP film.

3.2.2.7 Cloning LacI$^-$ Mutants

Mutant lacI$^-$ genes were cloned by recombination into the M13 derivative mRS81 essentially as described by Schaaper et al. (1985). F'lac were transferred from X^r strains (recA$^-$) to strain S90C (recA$^+$) as described in section 3.2.2.3. S90C sexductants were mixed with mRS81 at a multiplicity of infection of 0.1 in 1 ml LB, and incubated overnight with vigorous shaking at 37^o C. In this step bacterial F'lacI$^-$Z$^+$ recombined with M13 phage (lacI$^+$Z$^-\alpha$) to produce some lacI$^-$ Z$^+$ phage. Bacteria were pelleted by centrifugation, and 5 µl of the supernatant containing the phage removed to dilution tubes containing sodium borate. Ten µl of a 5 X 10^{-6} dilution of phage were added to 0.5 ml of an exponentially growing culture of NR9099 in 3 ml XGal top agar and plated onto minimal medium. Recombinant lacI$^-$Z$^+$ phage were identified by their blue colour on XGal after 2-3 days. Blue plaques were picked and purified by replating on NR9099. If no blue recombinants were recovered on the first plating, a larger number of phage were plated in subsequent experiments. Recombinant phage from an isolated blue plaque was amplified by overnight growth in 6 ml of NR9099 culture. Bacteria were pelleted by centrifugation at 5000 x g for 15 minutes. The supernatant (4.5 ml) was removed to Corex tubes containing 1.5 ml 2.5 M NaCl, 15 % polyethylene glycol 8000; the mixture was chilled on ice; and the phage precipitated by centrifugation at 10,000 x g for 30 minutes. Phage were taken up in phage phenol extraction buffer (100 mM Tris-HCl pH 8.0, 300 mM NaCl, 1 mM EDTA), transferred to 1.5 ml Eppendorf tubes, the DNA isolated by extraction with phenol/chloroform/isoamyl alcohol (PCI) (25:24:1)

(twice) and precipitated by the addition of 1/10th volume 3 M sodium acetate and 2 volumes of 95 % ethanol. The DNA was pelleted by centrifugation for 6 minutes in a microfuge, washed with 70 % ethanol and taken up in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) (Davis et al., 1986).

3.2.2.8 DNA Sequencing

LacI$^-$ mutant DNA was sequenced by the dideoxy chain termination method (Sanger et al., 1977; 1980) using [α-^{35}S]dATPαS incorporation. Dideoxy NTP mixtures were prepared to the following specifications: all 4 mixtures contained 7.5 mM DTT, 10 mM Tris-HCl pH 7.5, and 5 mM $MgCl_2$; the ddATP mix contained 0.06 mM ddATP, 0.037 mM dCTP, 0.037 mM dGTP, and 0.037 mM dTTP; the ddCTP mix contained 0.125 mM ddCTP, 0.0185 mM dCTP, 0.185 mM dGTP, 0.185 mM dTTP; the ddGTP mix contained 0.125 mM ddGTP, 0.185 mM dCTP, 0.0185 mM dGTP and 0.185 mM dTTP; and the ddTTP mix contained 1.2 mM ddTTP, 0.185 mM dCTP, 0.185 mM dGTP and 0.0185 mM dTTP. Oligonucleotide primers for the sequencing reactions were 14-mers complementary to positions 148-161, 215-228, 302-315, 450-463, 604-617, 745-758, 901-914, and 1049-1062 of the wild-type lacI gene (Farabaugh, 1978), and a 17-mer (universal primer) which anneals to positions 27-43 of the lacZ gene (Reznikoff and Abelson, 1978). Annealing reactions consisted of 3 µl DNA sample, 0.5 µl of the appropriate primer, and 1.5 µl annealing buffer (100 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 50 mM DTT) in a 10 µl reaction volume. Reaction tubes were placed in a heating block at 70° C for 10 minutes, were cooled to below 40° C over a period of 60-90 minutes, and the contents were then mixed with 6 µl of labelling

buffer (18 mM DTT, 1.8 µM ATP) containing 5 µCi [α-^{35}S]dATPαS (1000 Ci/mmol). To facilitate sequencing large numbers of DNA samples, primer extension reactions were carried out in 96 well microtitre dishes (V-bottom). A 3.5 µl aliquot of annealed sample containing the labelling buffer and radiolabel was dispensed in the bottom of 4 adjacent wells. Klenow polymerase was diluted 1:12 in polymerase dilution buffer (50 % glycerol, 25 mM Tris-HCl, pH 7.6). The diluted enzyme (1 µl) and 2 µl of the appropriate dideoxy NTP mix were pipetted onto opposite sides of a well. Reactions were begun simultaneously by spinning the microtitre dish in a modified household salad spinner for 15 seconds. The contents of the microtitre plate were then incubated in a 37° C sandbath for 20 minutes. The reactions were stopped by the addition of 5 µl sequencing dye mix (5 mM EDTA, 0.1 % bromophenol blue, 0.1 % xylene cyanol, 99 % formamide), and the DNA strands separated by heating for 10 minutes in a 70° C sandbath. The dishes were placed on ice and 2.5 µl of each reaction loaded onto a 8 % polyacrylamide sequencing gel. Gels were run in a BRL Model S2 sequencing apparatus, using an EC 6000 power supply at constant power (60 watts) for 2-4 hours with voltage and current settings of 1800 Volts and 40 milliamperes, respectively. Gels were dried and autoradiographed for 2-3 days using Kodak XAR-5 or RP film.

3.2.2.9 Statistical Analysis

Statistical analysis of the frequency distribution of mutations was based on the Chi-square test for goodness of fit (Malik and Mullen, 1973).

3.2.2.10 Computer Assisted Analysis of DNA secondary Structure

The stability of potential DNA secondary structures was evaluated using the computer program described by Zuker and Steigler (1981). The program "FOLD" is part of the Genetics Computer Group software package (Devereux et al., 1984) for VAX/VMS computers. The program finds the minimum free energy of an oligonucleotide secondary structure (modelled as RNA) using the thermodynamic parameters described by Freier et al. (1986).

3.3 RESULTS

3.3.1 Selection of lacI⁻ Mutants

Spontaneous and 1,8-NONP-induced lacI⁻ mutants were selected from each of four E. coli strains (wild type; pKM101; ΔuvrB; and pKM101, Δ uvrB) using the PGal selection method (Smith and Sadler, 1971). Two hundred and seventy-nine independent spontaneous mutants (48 from wild-type, 70 from pKM101, 92 from uvrB⁻, and 69 from pKM101, uvrB⁻) were selected. A total of 811 mutants (263 from wild type, 149 from pKM101, 159 from Δ uvrB, and 240 from pKM101, Δ uvrB) were selected following 1,8-NONP treatment. The mutation frequency for each strain is shown in Table 7.

Cultures treated with 1,8-NONP are expected to contain two populations of mutations: 1,8-NONP-induced mutations which would arise during the 15 minute treatment period, or during selection on PGal plates; and spontaneous mutations which could arise at any point during culture growth, treatment, or selection. The selection protocol used in these experiments, which combined short treatment period with the chemical, incubation in buffer, and direct plating onto selective media ensures that the 1,8-NONP-induced mutants are of independent origin (Burns et al., 1988a). The likelihood of a spontaneous mutant being present in the collection decreases as the mutational index (mutational frequency (treated)/mutational frequency (spontaneous)) increases. Therefore, a significant proportion of the mutants derived from NR6112, in which there was approximately a 2-fold increase in mutation frequency over control, and EE125 (5-fold increase) are likely to be of spontaneous origin. In contrast, few

Table 7. Selection of *lacI*$^-$ mutants following 1,8-NONP treatment.

	NR6112 (wild-type)	EE125 (pRM101)	NR6113 (Δ *uvrB*)	CM6114 (pRM101, Δ *uvrB*)
Mutation freq. (treated)[a]	11.3 (4.2)[b]	36.0 (12.1)	105 (30)	265 (71)
Mutation freq. (control)[a]	5.4 (2.8)	7.3 (2.3)	3.2 (1.6)	3.0 (1.6)
Mutational index[c]	2.1	5.0	33	88
Survival	82%	81%	75%	71%
Total # Mutants selected	263	149	159	240

Cultures were treated with 1,8-NONP in DMSO or with an equal volume of DMSO only (controls).

[a] Mutation frequencies are expressed as the number of *lacI*$^-$ mutants (as determined by the number of colonies which formed on PGal) per 10^{-6} survivors.

[b] Values given are means from several independent determinations; standard deviations are shown in parentheses.
The number of plates counted for treated cultures: NR6112-36 plates; EE125-24 plates; NR6113-24 plates; CM6114-36 plates.
The number of plates counted for untreated cultures: NR6112-12 plates; EE125-8 plates; NR6113-8 plates; CM6114-12 plates.

[c] The mutational index measures the increase in mutation frequency in 1,8-NONP treated cultures relative to controls.

of the CM6114 (88-fold increase) or NR6113 (33-fold increase) mutants are likely to be of spontaneous origin.

Detailed knowledge of the DNA sequence changes associated with spontaneously occurring lacI$^-$ mutations is useful in evaluating which mutations in treated cultures might be of spontaneous origin. The spectrum of spontaneous lacI$^-$ mutations in bacteria with wild-type backgrounds has been reported in the literature (Farabaugh et al., 1978; Schaaper et al., 1986; Fix et al., 1987). In addition, unpublished spectra generated in Barry Glickman's laboratory (York University, Toronto) from wild-type and pKM101 strains have been made available and appear here with permission. Although a spontaneous mutation spectrum from a uvrB$^-$ strain has not been published, Schaaper et al. (1987) have referred to unpublished results in stating that the distribution of spontaneous mutations in a uvrB$^-$ strain is similar to that in a wild-type strain. The spontaneous spectrum from a uvrB$^-$, pKM101 strain has not been examined. In order to provide some form of interlaboratory comparison with the wild-type and pKM101 backgrounds, and to obtain information regarding the spontaneous mutation spectrum in a $_{\Delta}$uvrB, and a pKM101, $_{\Delta}$ uvrB background, a small number of independent spontaneous mutants were selected in strains containing each of the 4 DNA repair backgrounds, and were partially characterized.

3.3.2 Identification of Hotspot Mutations Using Oligonucleotide Probes

3.3.2.1 +TGGC and -TGGC Mutations Among Spontaneous Mutants

The spontaneous mutation spectrum in the lacI gene is

dominated by mutations involving the loss (-TGGC) or addition (+TGGC) of 5'-T-G-G-C-3' from the sequence 5'-T-G-G-C-T-G-G-C-T-G-G-C-3' at positions 621-632. (Farabaugh et al.,1978; Schaaper et al., 1986). In the lacI gene the +TGGC and -TGGC mutations are frequently referred to as hotspot mutations (Farabaugh et al., 1978; Miller, 1978). LacI$^-$ mutants containing either of these mutations, or the wild-type sequence can be rapidly identified by hybridization with oligonucleotides complementary to the mutant or wild-type sequences using the method of Miller and Barnes (1986). Mutants were fixed onto nylon filters, incubated with the appropriate ^{32}P-labelled probe, washed at a probe specific temperature, and autoradiographed. An example of a representative experiment is given in Figure 19. In general, mutants giving a positive result with the +TGGC or -TGGC probes were not characterized further. However, eleven of these mutants (8 +TGGC mutants and 3 -TGGC mutants) were selected randomly, cloned into the M13 vector mRS81, and the DNA sequence of the region determined. In every instance, this analysis confirmed the assignments that had been made based on the hybridization analysis.

Table 8 shows the number of +TGGC and -TGGC mutations present in the spontaneous lacI$^-$ mutants selected from each of the 4 DNA repair backgrounds used in these studies. In the wild-type, pKM101, and ΔuvrB strains the +TGGC and -TGGC mutations constituted about 65 % of the total mutation spectrum; in these 3 strains +TGGC mutations were considerably more prevalent than -TGGC changes. In contrast, only 39 % of the mutations collected in the pKM101, ΔuvrB background were hotspot mutations with -TGGC changes predominating.

Figure 19. Oligonucleotide probing experiment.
S90C(F'lacI$^-$) strains were picked onto nylon filters and grown overnight. The DNA was fixed onto the filter and probed with with a ^{32}P-endlabelled oligonucleotide complementary to the -TGGC mutant sequence using the procedure described by Miller and Barnes, 1986. Following autoradiography, mutants containing the -TGGC mutation were identified by the hybridization pattern. Mutant 2-76 (Table 6) acted as a positive control for the -TGGC probe and is designated by an arrow (◄). Other probing controls which were included in every experiment are described in Table 6: Mutant 1-91 (circled) contained a +TGGC mutation; mutant 1-17 (W) contained the wild-type sequence at positions 621-632; and mutant 1-51 (*) contained a G:C => A:T transition at position 631.

Table 8. +TGGC and -TGGC mutations in spontaneous mutants.

a] The number of spontaneous lacI⁻ mutations involving the gain or loss of the sequence 5'-TGGC-3' from the sequence 5'-TGGCTGGCTGGC-3' at nucleotides 621-632. These assignments were made on the basis of oligonucleotide probing experiments.

Genotype	wild-type	pKM101	ΔuvrB	pKM101 ΔuvrB
Total Hotspot (%)	31 (65%)	47 (67%)	58 (63%)	27 (39%)
+TGGC	25	38	41	10
-TGGC	6	9	17	17
Total nonhotspot[a]	17	23	34	42
Total	48	70	92	69

[a] refers to mutations other than +TGGC and -TGGC mutations.

b] The contribution of +/- TGGC mutations to spontaneous mutations in E. coli wild-type and pKM101 DNA repair backgrounds as determined by J.Halliday, A.Gordon, and B.Glickman (York University, unpublished results used with permission). A summary of the entire spectra appears in Figure 19. Values given are a percentage of the total number of mutants characterized.

Genotype	wild-type	pKM101
Total Hotspot	70	61
+TGGC	56	45
-TGGC	14	16
Total Nonhotspot[a]	30	39
Total # mutants	726	193

[a] refers to mutations other than +TGGC and -TGGC mutations.

The only complete, sequenced spontaneous mutation spectrum in the lacI gene that has appeared in the literature (Schaaper et al., 1986) was obtained from a wild-type DNA repair background. Of 169 lacI⁻ mutants characterized in that study, 69 % were +TGGC or -TGGC mutants. The +TGGC/-TGGC ratio was 4.3 : 1. A second study (Farabaugh et al., 1978), also using a wild-type DNA repair background, employed mainly genetic methods and examined 140 lacI⁻ mutants. The results were virtually identical: 67 % +TGGC and -TGGC mutants, with a +TGGC/-TGGC ratio of 4.3 : 1. An extremely comprehensive study of spontaneous mutations in a wild-type DNA repair background has been completed by Halliday and Glickman (Table 8b; Table 19; unpublished results used with permission): of 726 lacI⁻ mutants whose sequence has been determined, 70 % were +TGGC and -TGGC mutants, with a +TGGC/-TGGC ratio of 4 : 1. The spontaneous mutation spectrum in a strain containing the plasmid pKM101 (Table 8b; Table 19; Gordon and Glickman, unpublished results used with permission) has 61 % +TGGC and -TGGC mutations which is slightly lower than the wild-type background; the +TGGC/-TGGC ratio (2.8 : 1) is also lower.

The number of +TGGC and -TGGC mutations in the spontaneous mutant collections in three of the strains (wild type, pKM101, and ΔuvrB) examined here are in agreement with the studies cited above. Of 48 mutants selected from a wild-type background, 65 % contained the hotspot mutations with a +TGGC/-TGGC ratio of 4.1 : 1. Seventy mutants in a pKM101 background were selected for analysis, of which 67 % contained hotspot mutations with a ratio of 4.3 : 1. In the

uvrB⁻ strain 92 mutants were selected yielding 63 % hotspot mutations with a +TGGC/-TGGC ratio of 2.4 : 1. A statistical comparison (Chi-square test; 2 degrees of freedom) of the spectra in Tables 8a and 8b shows that the distribution of mutation (+TGGC, -TGGC, nonhotspot) in the wild-type and plasmid pKM101-containing strains is not significantly different in the two collections presented in these tables. The +TGGC/-TGGC ratio (2.4 : 1) observed in the uvrB⁻ strain is slightly lower than that in the wild-type spectrum. However, this difference is not statistically significant, consistent with the observation of Schaaper et al. (1987) that the spontaneous mutation spectrum is not altered by a deficiency in excision repair. The spontaneous mutational spectrum in the pKM101, uvrB⁻ strain differs considerably from that in the other three strains. Only thirty-nine percent are +/-TGGC mutations with a +TGGC/-TGGC ratio of 0.58 : 1. This distribution is significantly ($P<0.001$) different from that observed in wild type, pKM101 or uvrB⁻ strains. No further experiments have been undertaken to determine the DNA sequence changes of spontaneous mutants which did not contain +TGGC or -TGGC mutations.

3.3.2.2 +TGGC and -TGGC Mutations Following 1,8-NONP Treatment

All mutants isolated from 1,8-NONP treated cultures were analyzed by oligonucleotide hybridization to determine whether they contained +TGGC or -TGGC mutations. The results are given in Table 9. Sixty-three percent of the 263 mutants isolated from the wild-type DNA repair strain (NR6112) contained +TGGC (49 %) or -TGGC (14%)

Table 9. +TGGC and -TGGC mutations following 1,8-NONP treatment

Strain	NR6112 (wild-type)	EE125 (pKM101)	NR6113 (ΔuvrB)	CM6114 (pKM101, ΔuvrB)
Mutational Index	2.1	5.0	33	88
Total +/-TGGC	170 (63%[a])	61 (41%)	19 (12%)	6 (2.5%)
+TGGC	129	45	15	4
-TGGC	41	16	4	2
Total # mutants	263	149	160	240

Values are the number of +/- TGGC mutations among the lacI⁻ mutants recovered from the 4 E. coli strains following 1,8-NONP treatment. Assignments are based on oligonucleotide probing experiments and in some cases have been confirmed by DNA sequencing.

[a] Values in parentheses are the percentage of mutants containing a +TGGC or -TGGC mutation.

mutations, with a +TGGC/-TGGC ratio of 3.2 : 1. In this strain, a very small (2.1-fold) increase in mutation frequency was calculated following 1,8-NONP treatment. The collection of mutations recovered from EE125 (pKM101) following 1,8-NONP treatment contained 41 % hotspot mutations with a +TGGC/-TGGC ratio of 2.8 : 1. Of 159 mutants selected from strain NR6113 (uvrB$^-$) which had been treated with 1,8-NONP, only 12 % contained hotspot mutations, with a +TGGC/ -TGGC ratio of 3.8 : 1. The pKM101, uvrB$^-$ strain (CM6114) exhibited the greatest mutational response to 1,8-NONP (88 X the spontaneous mutation frequency); of the 240 mutants collected, only 2.5 % contained +TGGC or -TGGC mutations. A general trend is obvious from these results: the contribution of +TGGC and -TGGC mutations to the overall mutational spectrum decreased in those strains which are most sensitive to 1,8-NONP.

3.3.3 Deletion Mapping of LacI$^-$ Mutants and the Dominance Test

Deletion mapping studies were done to localize the mutation to a particular region of the gene so that a minimum of DNA sequencing would be necessary. Six E. coli strains harboring tonB deletions extending various distances into the chromosomal lacI gene from the 5'-end (Schmeissner et al., 1977b) were used in these studies (Section 3.2.2.5). The endpoints for these deletions produced a low resolution deletion map consisting of 6 intervals. More than 92 % of the mutants examined in this study were ultimately found to contain a change in DNA sequence in the interval to which they were first assigned following the mapping studies. Thus, the mapping results generally made it possible to obtain the identity of

the mutation by a single sequencing run initiated by an appropriate oligonucleotide primer.

The initial 180 base pairs of the first deletion zone encode the DNA-binding domain of the lactose repressor. Mutants in this region can be identified by a simple dominance test (Miller, 1972). The F'lacpro of interest was mated into the strain CSH52 (RecA$^-$) which contains the wild-type repressor. F'LacI$^-$ genes containing a mutation within the first 180 base-pairs generally encode a repressor which is dominant to the wild-type repressor. This is due to the formation of mixed (wild-type and mutant) tetramers which are functionally lacI$^-$. These mutations are termed I^{-d} (Miwa and Sadler, 1977; Miller, 1978) and are easily identified by the blue colour of CSH52(F'lacI^{-d}) sexductants on XGal plates. Any mutations within the lac regulatory region (O^c mutations) will also be scored as dominant mutants in this test. In contrast, mutations in the aggregation domain will not form mixed tetramers and will therefore be recessive. Mutations which result in premature termination of translation within the first 60 amino acids are also detected as dominant in this test. This is due to reinitiation of translation at amino acids 23, 42, and 62 (Miller, 1978; Cone and Steege, 1985a; 1985b). Thus missense mutations, nonsense mutations, and frameshift, duplication or deletion mutations resulting in the gain or loss of 3n or 3n-1 bases will all be dominant if they occur within the first 180 bases of the coding portion of the lacI gene. Mutations resulting from changes in the reading frame of 3n+1 bases, or deletions whose endpoints are downstream from the first 180 base pairs, will not reinitiate at

amino acids 23, 42, or 62 and will be recessive. Using this test one hundred and thirty-eight mutants were identified which exhibited the I^{-d} phenotype and which mapped to the first deletion interval. These were sequenced using an oligonucleotide primer complementary to positions 215-228 of the wild-type lacI gene.

3.3.4 Cloning LacI$^-$ Mutations

LacI$^-$ mutations which were not +TGGC or -TGGC mutations (i.e. nonhotspot mutations) were cloned into the M13 vector mRS81 as described in sections 3.1.1.1 and 3.2.2.7 (Schaaper et al., 1985). About 5 X 10^4 phage were plated on the indicator strain NR9099. This generally resulted in the production of between 1 and 25 blue plaques. The majority of the lacI$^-$ mutations in this study were recovered onto vector mRS81 on the first attempt. For those mutations which did not yield blue recombinants, larger numbers of phage were plated. Only 38 of the nonhotspot lacI$^-$ mutations proved refractory to repeated cloning attempts (10 in NR6112, 8 in EE125, 6 in NR6113, and 14 in CM6114). Following DNA sequence analysis, it was possible to evaluate the efficiency with which various classes of mutation were recovered onto mRS81. Point mutations (single base frameshifts and base substitutions) and small deletions (2-10 nucleotides) were generally recovered with a frequency of 5-10 X 10^{-4}. Deletions of 11-20 nucleotides were recovered with an average frequency of about 1 X 10^{-4}. Deletions of greater than 20 nucleotides were recovered with a frequency of 1-5 X 10^{-5}. The observation that recombinational recovery is reduced as the extent of the physical damage increases has also been reported by Schaaper et

al. (1986).

It is likely that the 38 mutations that were not recovered into mRS81 contained large deletions or additions which reduced the efficiency of recombination to below the detection limit. Some lacI$^-$ mutants containing deletions that were not recovered onto mRS81 were partially characterized by their behaviour in the deletion mapping, dominance, and oligonucleotide hybridization studies discussed in sections 3.3.2 and 3.3.3. a) Some deletions which extend through the middle of the lacI gene will eliminate sequences which hybridize with the +TGGC, -TGGC or wild-type probes, and will map to one of the first three deletion intervals. b) Mutants containing deletions which extend from near the end of the lactose repressor gene into the regulatory region of lacZ will be operator constitutive mutants (O^C) and since repressor will not be capable of binding to the operator they will yield blue colonies when transferred into CSH52 and plated on XGal. Deletions such as those described in a) above, which are entirely within the lacI gene, have previously been classified as class I deletions; while the deletions described in b), which extend from within the repressor into the lac regulatory sequences, have been classified as class II deletions (Schaaper et al., 1986). Twenty out of the 38 mutations which were not recovered meet one of the above criteria as described in Table 10.

The remaining 18 mutants (4 in NR6112, 3 in EE125, 1 in NR6113, and 10 in CM6114) might contain large class I deletions within the lacI gene which do not eliminate bases between positions 617 and 636, or deletions which extend outside the portion of the

Table 10. Partial characterization of probable *lacI* deletions which were not recovered into mRS81.

Strain	NR6112 (wild-type)	EE125 (pKM101)	NR6113 (Δ*uvrB*)	CM6114 (pKM101 Δ*uvrB*)
# unrecovered	10	8	6	14
class I deletions	1	2	2	1
class II deletions	5	3	3	3

The criteria for making these assignments are based on genetic and probing studies as described in the text.

lactose operon which resides in mRS81 (and subsequently abolish recovery through homologous recombination). Large duplications or the insertion of transposable elements into the lacI gene are other mutational events which would be expected to reduce the efficiency of recombination onto mRS81.

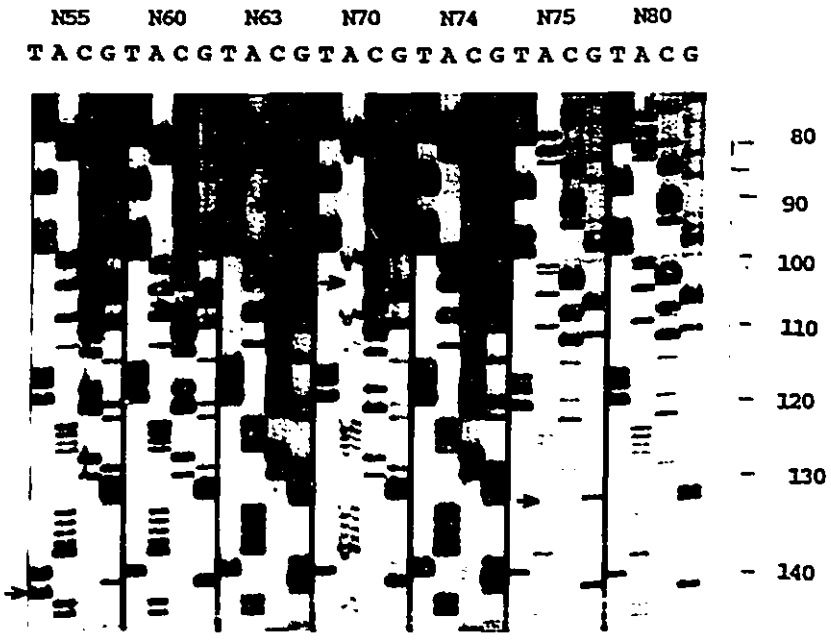
3.3.5 DNA Sequence Analysis

DNA sequencing was accomplished using the dideoxy chain termination method (Section 3.2.2.8). In a typical experiment, the DNA samples from 15 to 20 mutants were sequenced in a parallel series of reactions initiated by a particular primer. The DNA sequence was revealed by running the products of the sequencing reactions in adjacent lanes on an 8 % polyacrylamide gel. This arrangement facilitated reading of the mutant sequence since the positions of most mutations were flanked on either side by wild-type sequences. This is illustrated in Figure 20. The DNA sequences were compared to the known wild-type sequence of the lacI gene (Farabaugh, 1978), and the regulatory region between lacI and lacZ (Reznikoff and Abelson, 1978) (Figure 21).

3.3.5.1 Base Substitutions

A total of 142 base substitution mutations (11 in NR6112, 39 in EE125, 12 in NR6113 and 80 in CM6114) were recovered at 77 different sites. These are listed in Table 11. A summary of this data according to the type of base substitution is given in Table 12.

Only 4.4 % of the characterized mutations collected from strain NR6112 which had been treated with 1,8-NONP involved base substitution mutations; all occurred at G:C base pairs. The ratio of



The wild type sequence for the area shown is as follows (reading from top to bottom):

```
71          80          90          100         110         120
|           |           |           |           |           |
GTC TCT TAT CAG ACC GTT TCC CGC GTG GTG AAC CAG GCC AGC CAC GTT TCT

          130         140         150
          |           |           |
GCG AAA ACG CGG GAA AAA GTG GAA GCG GCG
```

Figure 20. DNA sequencing of lacI⁻ mutations.
DNA sequencing reactions were carried out as described in the text, and the products were loaded onto an 8 % polyacrylamide gel. When reading the DNA sequences it was generally possible to locate the position of the mutation quite rapidly since adjacent sequences were usually wild-type. The mutations are denoted by the arrows (←). The DNA samples were obtained from the following mutants (from left to right): N55 contained a G => T transversion at position 143; N60 contained a -1 (G) frameshift at position 108-109; N63 contained a -1 (C) frameshift at position 90-92 (in the photo the contrast is poor at the position of this mutation; however, it can be clearly seen that the distance between T(89) and T(96) is reduced by one nucleotide); N70 contained a A => C transversion at position 105; N74 contained a G => T transversion at position 221 and therefore the entire sequence shown in this figure is wild-type; N75 contained a -1 (G) frameshift at position 132-134; N80 contained a C => A transversion at position 75.

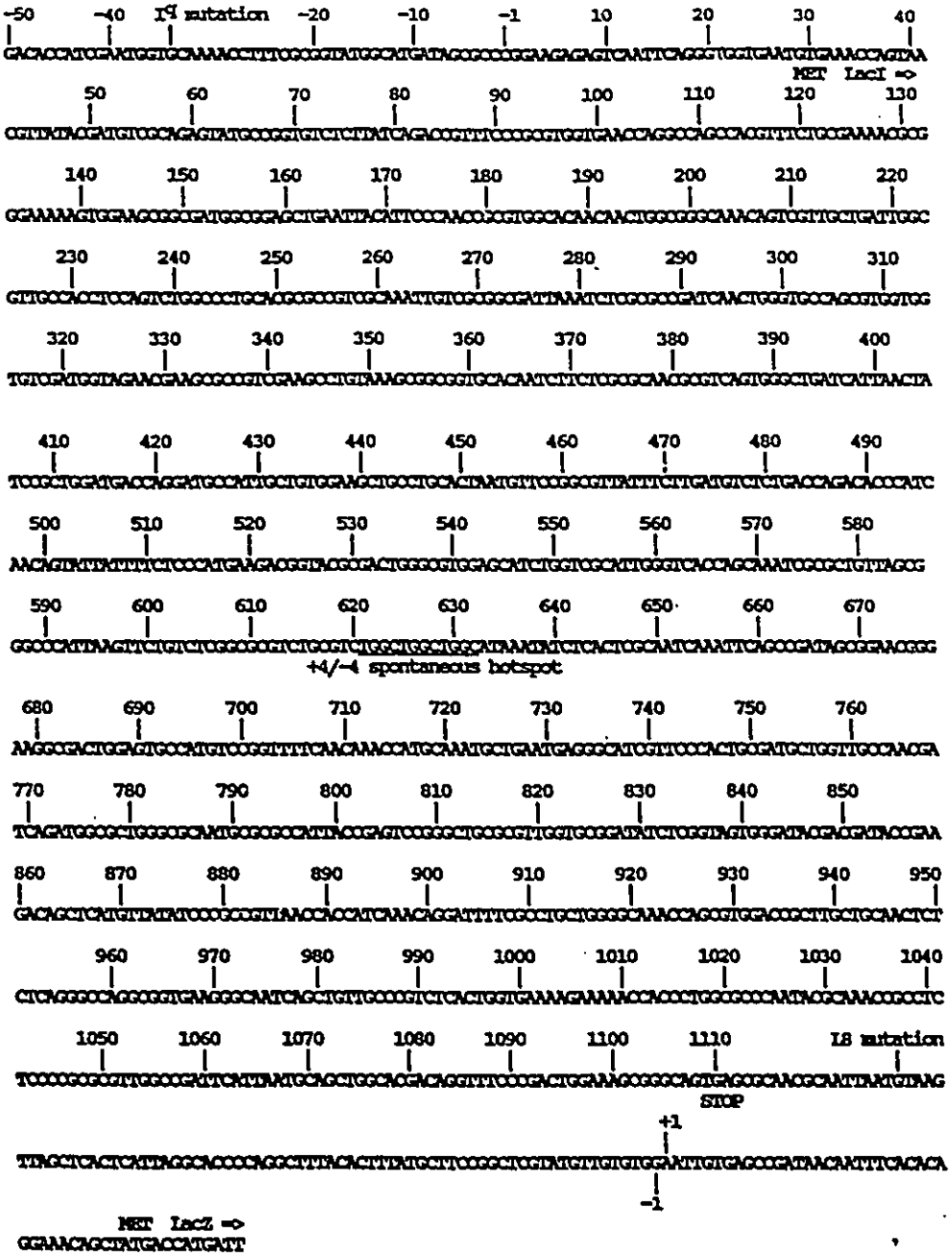


Figure 21. Wild-type sequence of the lacI gene.
This figure shows the sequence of the lacI gene, the lac regulatory region, and the beginning of the lacZ gene. Numbering is according to Farabaugh (1978), and Reznikoff and Abelson (1978). The positions of the I^q (Muller-Hill et al., 1969) and L8 (Scaife and Beckwith, 1967) promotor mutations, and the +4/-4 spontaneous mutation hotspot is also shown.

Table 11. Base substitution mutations recovered from E. coli strains exposed to 1,8-NONP. The table continues on the following page.

Site	Change	Amino Acid Change	Sequence[a]	NR6112 (wild-type)	EE125 (pRM101)	NR6113 (ΔuvrB)	CM6114 (pRM101ΔuvrB)
49	C - A	Tyr ⇒ Oc3	ACATC G TATAA	0	1	0	0
53	G - T	Val ⇒ Phe	ACGAT G TCGCA	0	2	0	0
54	T - G	Val ⇒ Gly	CTGCG A CATCG	0	1	0	0
54	T - C	Val ⇒ Ala	CTGCG A CATCG	0	1	0	0
56	G - A	Ala ⇒ Thr	ATGTC G CAGAG	0	0	1	0
57	C - A	Ala ⇒ Glu	ACTCT G CGACA	0	2	0	2
59	G - T	Glu ⇒ Am2	TCGCA G AGTAT	0	0	0	1
66	C - A	Ala ⇒ Asp	CACCG G CATAC	0	0	0	1
72	T - A	Val ⇒ Asp	AAGAG A CACCG	0	1	0	0
75	C - A	Ser ⇒ Tyr	GATAA G AGACA	0	0	0	2
80	C - A	Gln ⇒ Lys	GGTCT G ATAAG	0	0	0	3
84	C - A	Thr ⇒ Asn	AAACG G TCTGA	1	0	1	0
86	G - C	Val ⇒ Leu	AGACC G TTTCC	0	0	0	1
89	T - A	Ser ⇒ Thr	GCGGG A AACGC	0	0	0	1
90	C - A	Ser ⇒ Tyr	CGCGG G AAACG	0	1	0	0
90	C - T	Ser ⇒ Phe	CGCGG G AAACG	0	0	0	1
92	C - A	Arg ⇒ Thr	CACGC G GGAAA	0	0	0	2
93	G - A	Arg ⇒ His	TTCCC G CGTGG	1	0	1	0
93	G - T	Arg ⇒ Leu	TTCCC G CGTGG	0	3	0	1
102	A - T	Asn ⇒ Ile	GGTGA A CCAGG	0	0	0	1
104	C - T	Gln ⇒ Amb	GGCCT G GTTCA	1	1	1	0
104	C - A	Gln ⇒ Lys	GGCCT G GTTCA	0	0	0	1
105	A - C	Gln ⇒ Pro	GAACC A GGCCA	0	0	0	1
107	G - A	Ala ⇒ Thr	ACCAG G CCAGC	0	1	0	0
110	A - T	Ser ⇒ Cys	AGGCC A GCCAC	0	0	0	1
111	G - A	Ser ⇒ Asn	GGCCA G CCACG	1	0	0	0
116	G - T	Val ⇒ Phe	GCCAC G TTTCT	0	1	1	1
120	C - A	Ser ⇒ Tyr	TCGCA G AAACG	0	0	0	2
134	G - T	Glu ⇒ Ochre	CGCGG G AAAAA	0	0	0	1
140	G - T	Val ⇒ Leu	AAAAA G TGGAA	0	0	1	0
143	G - T	Glu ⇒ Ochre	AAGTG G AAGCG	0	0	0	3
150	C - A	Ala ⇒ Glu	CCATC G CCGCT	0	1	0	0
158	G - T	Glu ⇒ Am7	TGGCG G AGCTG	0	2	0	0
169	C - A	Tyr ⇒ Oc8	GGAAT G TAATT	0	0	0	1
174	C - A	Pro ⇒ His	GGTTG G GAATG	0	0	0	2
174	C - G	Pro ⇒ Arg	GGTTG G GAATG	0	0	0	1
180	G - C	Arg ⇒ Pro	CAACC G CGTGG	1	0	0	0
185	G - A	Ala ⇒ Thr	GCGTG G CACAA	0	1	0	0
185	G - C	Ala ⇒ Pro	GCGTG G CACAA	0	1	0	0
185	G - T	Ala ⇒ Ser	GCGTG G CACAA	0	0	0	1
186	C - A	Ala ⇒ Glu	GTTGT G CCACG	1	0	0	2
195	T - A	Leu ⇒ Gln	CCGCC A GTTGT	0	0	0	1
200	G - T	Gly ⇒ Cys	TGGCG G GCAAA	0	0	0	1
201	G - T	Gly ⇒ Val	GGCGG G CAAAC	0	0	0	2
203	A - T	Lys ⇒ Ochre	CGGGC A AACAG	0	0	0	1
210	C - A	Ser ⇒ Am10	GCAAC G ACTGT	1	0	0	4
221	G - T	Gly ⇒ Cys	TGATT G GCGTT	0	0	0	3

Site	Change	Amino Acid Change	Sequence[a]	NR6112 (wild-type)	EE125 (pKM101)	NR6113 (ΔuvrB)	CM6114 (pKM101ΔuvrB)
222	G - A	Gly ⇒ Asp	GATTG G CGTTG	1	0	0	0
222	G - T	Gly ⇒ Val	GATTG G CGTTG	0	0	0	1
228	C - A	Ala ⇒ Asp	AGGTG G CAACG	0	2	0	0
258	C - A	Ser ⇒ Am12	TTTGC G ACGGC	0	0	0	2
260	C - T	Gln ⇒ Oc11	AATTT G CGACG	0	0	1	0
270	C - A	Ala ⇒ Glu	TCGCC G CGACA	0	0	0	1
318	C - A	Ser ⇒ Am13	CCATC G ACACC	0	0	0	4
326	G - T	Glu ⇒ Oc14	TGGTA G AACGA	0	1	0	1
341	G - T	Glu ⇒ Oc15	GCGTC G AAGCC	0	0	0	4
381	G - A	Arg ⇒ His	GCAAC G CGTCA	0	0	0	1
437	G - T	Glu ⇒ Oc19	CTGTG G AAGCT	1	0	0	0
487	G - T	Gln ⇒ His	GACCA G ACACC	0	1	0	0
518	G - T	Glu ⇒ Oc20	CCCAT G ANGAC	0	0	0	1
528	C - A	Thr ⇒ Lys	CTCGC G TACCG	0	0	0	2
537	G - A	Gly ⇒ Asp	ACTGG G CGTGG	0	0	0	1
623	G - T	Ala ⇒ Ser	GTCTG G CTGGC	0	1	0	0
677	G - T	Glu ⇒ Oc26	AACGG G AAGGC	0	0	0	1
693	C - A	Ala ⇒ Asp	GAGTG G CATGT	0	1	0	1
702	G - A	Gly ⇒ Asp	GTCCG G TTTTC	0	0	1	0
731	G - T	Glu ⇒ Am25	TGAAT G AGGGC	0	1	0	1
750	C - A	Ala ⇒ Glu	GCATC G CAGTG	0	0	0	3
776	G - C	Ala ⇒ Pro	AGATG G CGCTG	0	0	1	0
782	G - T	Gly ⇒ Cys	CGCTG G GCGCA	0	0	1	0
783	G - A	Gly ⇒ Asp	GCTGG G CGCAA	1	0	0	0
783	G - T	Gly ⇒ Val	GCTGG G CGCAA	0	1	0	1
795	C - A	Ala ⇒ Tyr	TAATG G CGCGC	0	0	0	2
803	G - T	Glu ⇒ Am27	TTACC G AGTCC	0	1	0	0
834	C - A	Ser ⇒ Am28	CTACC G AGATA	0	1	0	0
842	G - A	Gly ⇒ Arg	TAGTG G GATAC	0	0	2	0
842	G - T	Gly ⇒ Opal	TAGTG G GATAC	0	2	0	0
843	G - T	Gly ⇒ Val	AGTGG G ATACG	0	0	0	2
857	G - T	Glu ⇒ Oc31	ATACC G AAGAC	0	1	0	0
867	C - A	Ser ⇒ Oc32	AACAT G AGCTG	1	1	0	3
896	A - T	Lys ⇒ Ochre	CCATC A AACAG	0	0	0	1
928	C - A	Ser ⇒ Arg	TCCAC G CTGGT	0	1	0	0
939	T - A	Leu ⇒ Am32	GCAGC A AGCGG	0	0	0	1
959	C - T	Gln ⇒ Am34	CGCCT G GCCCT	0	0	0	1
993	C - A	Ser ⇒ Oc36	CCAGT G AGACG	0	2	0	1
1005	G - T	Arg ⇒ Ile	GAAAA G AAAAA	0	1	0	0
1013	A - C	Thr ⇒ Pro	AAACC A CCCTG	0	1	0	0

This table shows the position, type of changes and surrounding sequence of the base substitution mutations, as well as the resultant amino acid changes. The sequence shown is that of the purine containing strand, and is read 5' => 3'. Nonsense mutations are classified according to type (amber (UAG), ochre (UAA), and opal (UGA)), and when possible are numbered according to Miller et al. (1978). The values given are independent occurrences of base substitutions at each site. The numbering of the position of the mutation is according to Farabaugh (1978).

[a] The sequence is read 5'=> 3' on strand containing G or A.

Table 12. Distribution of transversions and transitions in strains treated with 1,8-NONP.

Strain	NR6112 (wild-type)	EE125 (pKM101)	NR6113 (ΔuvrB)	CM6114 (pKM101 ΔuvrB)
Transversions				
G:C => T:A	5	31	4	66
G:C => C:G	1	1	1	2
A:T => T:A	0	1	0	7
A:T => C:G	0	2	0	1
Transitions				
G:C => A:T	5	3	7	4
A:T => G:C	0	1	0	0
Total	11	39	12	80
% sequenced mutants[a]	4.4%	30%	8.0%	37%
% nonhotspot mutations[b]	14%	57%	9.2%	38%
% at G:C sites[c]	100%	90%	100%	91%
Missense mutations	7	25	10	48
Nonsense mutations	4	14	2	32

[a] the percentage of total characterized mutants which are base substitutions.
[b] the percentage of characterized nonhotspot mutations (i.e mutations other than +TGGC and -TGGC mutations) which are base substitutions.
[c] the percent of base substitution mutations occurring at G:C base pairs.

transversions to transitions was 1.2 : 1. A similar distribution of events was observed in the mutations recovered from NR6113. In this strain, 8 % of the characterized mutations were base substitutions, all of which occurred at G:C base pairs with a transversion to transition ratio of 0.7 : 1. In strains NR6112 and NR6113 base substitution mutations constituted a very minor proportion of the entire mutational spectrum following 1,8-NONP treatment.

A marked increase in both the proportion of base substitution events, and the ratio of transversions to transitions was observed with E. coli strains containing the plasmid pKM101 (EE125 and CM6114). Thirty percent of the lacI⁻ mutations recovered from EE125 following 1,8-NONP treatment contained base substitutions. Ninety percent of these occurred at G:C sites. The ratio of transversions to transitions was 8.8 : 1. Eighty percent of EE125 base substitutions were G:C => T:A transversions. Thirty-seven percent of CM6114 lacI⁻ mutations contained base substitutions mutations. Ninety percent of these occurred at G:C sites and the ratio of transversions to transitions was 19 : 1. Eighty-two percent of the base substitutions recovered from CM6114 were G:C => T:A transversions.

A large number of nonsense mutations in the lacI gene can be suppressed in bacterial strains harboring suppressor tRNA species (reviewed in Miller, 1978). This provides the basis for a simple genetic test (section 3.2.2.4) to confirm the assignment of nonsense mutations. Fifty-two of the base substitution mutations characterized following 1,8-NONP treatment were nonsense mutations

(23 amber (UAG), 27 ochre (UAA), and 2 opal (UGA)). Of these 52 mutants, 42 occurred at sites which have been shown to be suppressible by the tRNA species Su3 (supF), SuB (supB), or SuC (supC) (Miller et al., 1977; Schmeissner et al., 1977a; Coulondre et al., 1978; Miller et al., 1978, Miller et al., 1979). The ability of all lacI$^-$ mutations to be suppressed by the insertion of the amino acids glutamine (SuB) and tyrosine (Su3, SuC) was determined. Repressor activity was restored only to those 42 mutants containing suppressible mutations.

3.3.5.2 Frameshift Mutations

The term "frameshift" applies to any gain or loss of bases which results in a change of reading frame during translation. However, the use of this term here will be restricted to mutations involving the gain or loss of one or two bases. Loss of 3 or more bases will be referred to as deletions; reiteration of 3 or more bases as duplications; and nonduplicative insertion of 3 or more bases as additions.

A total of 295 frameshift mutations (40 in NR6112, 23 in EE125, 113 in NR6113, and 119 in CM6114) at 76 different sites were recovered from the 4 E. coli strains treated with 1,8-NONP. These are described in detail in Table 13. The distribution of these mutants is summarized in Table 14.

In all strains the vast majority of frameshift mutations involved the loss of bases; only 7 frameshift mutations out of 295 involved the gain of a base. Ninety-six percent of all frameshift mutations occurred at G:C base pairs (279 losses; 3 gains). Only 13

Table 13. Frameshift mutations recovered from E. coli strains treated with 1,8-NONP. The table continues on the next page.

Site	Change	Sequence[a]	NR6112 (wild-type)	EE125 (pKM101)	NR6113 (Δ uvrB)	CM6114 (pKM101 Δ uvrB)
1 Base Frameshift						
66-67	-C	CACC GG CATA	0	0	0	1
90-92	-C	ACGC GGG AAAC	1	4	8	10
90-92	+C	ACGC GGG AAAC	1	0	0	0
108-109	-C	GGCT GG CCTG	0	0	1	1
112-113	-C	ACGT GG CTGG	0	0	2	3
132-134	-G	ACGC GGG AAAA	0	0	0	3
132-134	+G	ACGC GGG AAAA	0	1	0	1
135-139	-A	CGGG AAAAA GTGG	1	0	0	0
135-139	+A	CGGG AAAAA GTGG	0	0	1	1
142-143	-G	AAGT GG AAGC	0	0	5	1
148-149	-G	AAGC GG CGAT	0	0	2	0
171-172	-T	TGGG AA TGTA	0	0	0	1
173-175	-C	GGTT GGG AATG	3	0	1	4
186	-C	TTGT G CCAC	0	0	2	1
187	+A	TGGC A CAAC	0	1	0	0
188	-C	TGTT G TGCC	0	0	0	1
189-190	-A	GCAC AA CAAC	3	0	0	0
199-201	-G	TGGC GGG CAAA	0	0	4	1
202	-C	GTTT G CCCG	0	0	0	1
221-222	-G	GATT GG CGTT	2	0	0	0
228-229	-C	AGGT GG CAAC	1	0	0	0
234-235	-C	GACT GG AGGT	0	0	4	4
241-242	-G	GTCT GG CCCT	1	1	0	0
243-245	-C	TGCA GGG CCAG	0	1	5	3
298-300	-G	AACT GGG TGCC	0	0	2	2
303-304	-C	CGCT GG CACC	0	0	1	2
358-359	-G	CGGC GG TGCA	0	0	1	0
385	-C	CACT G ACGC	0	1	0	0
389-391	-G	CAGT GGG CTGA	0	2	2	3
412-413	-G	CGCT GG ATGA	1	0	6	1
421-422	-G	ACCA GG ATGC	0	0	3	2
426-427	-C	CAAT GG CATC	0	1	0	0
436-437	-G	CTGT GG AAGC	0	0	1	0
458-459	-C	CGCC GG AACA	0	0	1	0
473	-G	TCTT G ATGT	0	0	1	0
484-485	-C	GTCT GG TCAG	1	0	0	0
491-493	-C	TGAT GGG TGTC	1	1	6	7
496	-C	TGTT G ATGG	0	0	0	1
513-515	-C	TCAT GGG AGAA	0	1	1	1
524-525	-G	AGAC GG TACG	1	1	0	0
535-537	-G	GACT GGG CGTG	0	0	1	0
559-561	-G	CATT GGG TCAC	1	0	0	0
586-588	-G	TAGC GGG CCCA	1	0	3	2

Site	Change	Sequence[a]	NR6112 (wild-type)	EE125 (pKM101)	NR6113 (ΔuvrB)	CM6114 (pKM101 ΔuvrB)
589-591	-C	TATT GGG CCCG	1	1	0	4
604	+C	CCGA G ACAG	1	0	0	0
664	-G	AGCC G ATAG	0	0	1	0
670-671	-G	TAGC GG AACG	0	0	0	1
675-677	-G	GAAC GGG AAGG	0	1	6	1
680-681	-G	GGAA GG CGAC	0	0	0	1
687-688	-G	GACT GG AGTG	0	0	2	0
693-694	-C	ACAT GG CACT	0	0	1	0
733-735	-G	ATGA GGG CATC	0	0	5	2
743-745	-C	CAGT GGG AACG	1	1	0	10
751	-G	CTGC G ATGC	0	0	1	0
756-758	-G	TGCT GG TTGC	0	0	0	2
781-783	-G	CGCT GGG CGCA	2	0	5	5
809-811	-G	GTCC GGG CTGC	2	0	3	1
841-843	-G	TAGT GGG ATAC	4	1	1	1
851	-G	CGAC G ATAC	0	0	1	0
877-879	-C	CGGC GGG ATAT	0	0	2	9
888-889	-C	TGGT GG TTAA	0	0	0	1
891-892	-C	TGAT GG TGGT	0	0	0	1
896-898	-A	CATC AAA CAGG	1	0	0	1
916-919	-G	TGCT GGGG CAAA	3	0	14	12
927	-G	ACCA G CGTG	0	1	0	0
955-957	-G	CTCA GGG CCAG	0	0	1	1
970-972	-G	TGAA GGG CAAT	1	0	2	4
985	-G	TGTT G CCCG	0	0	0	2
986-988	-C	AGAC GGG CAACA	1	0	2	0
1006-1010	-A	AAAG AAAAA CCAC	0	2	0	0
1006-1010	+A	AAAG AAAAA CCAC	1	0	0	0
1011-1012	-C	GGGT GG TTTT	0	0	0	1
2 base frameshifts						
160-161	-GC	CGGA GC TGAA	1	0	0	0
250-253	-CG	TGCA CGCG CCGT	1	0	0	2
575-578	-GC	AATC GCGC TGTT	1	0	0	1
790-795	-GC	CAAT GCGCGC CATT	0	0	2	0

This table shows the position, type of change and sequence surrounding the mutated site of all frameshift mutations isolated following 1,8-NONP treatment. Numbering is according to Farabaugh (1978).

[a] The sequence given is taken from the purine containing strand. For -2 frameshifts the sequence is taken from the nontranscribed strand.

Table 14. Distribution of frameshift mutations recovered from E. coli strains following 1,8-NONP treatment.

Strain	NR6112 (wild-type)	EE125 (pKM101)	NR6113 (ΔuvrB)	CM6114 (pKM101 ΔuvrB)
-1 Frameshifts	34	21	110	115
-2 Frameshifts	3	0	2	3
+1 Frameshifts	3	2	1	1
Total	40	23	113	119
% sequenced mutants[a]	16%	18%	76%	55%
% nonhotspot mutations[b]	51%	33%	88%	56%
% at G:C base pairs[c]	85%	87%	99%	99%

[a] the percentage of total characterized mutants which are frameshift mutations.
[b] the percentage of characterized nonhotspot mutations (i.e mutations other than +TGGC and -TGGC mutations) which are frameshift mutations.
[c] the percent of frameshift mutations occurring at G:C base pairs.

frameshift mutations occurred at A:T base pairs (9 losses; 4 gains).

In NR6112, 16 % of the mutations recovered following 1,8-NONP treatment were frameshift mutations. Eighty-five percent of these events occurred at G:C base pairs. Frameshift events accounted for the majority (51 %) of nonhotspot mutational events in the wild-type DNA repair background. In EE125 (pKM101), frameshifts accounted for 18 % of the mutants and occurred primarily at G:C base pairs (87 %). Thirty-three percent of nonhotspot mutations were frameshifts.

Frameshift mutation was the major mutational pathway in 1,8-NONP-treated bacteria which were deficient in nucleotide excision repair capability (NR6113 and CM6114). Seventy-six percent of the characterized mutations in NR6113 mutants were frameshifts, accounting for fully 88 % of all nonhotspot mutations. Fifty-five percent of the characterized mutations recovered from CM6114 which had been treated with 1,8-NONP were frameshift mutations. A combined total of 232 frameshift mutations were recovered from NR6113 and CM6114. Of these, only 4 occurred at A:T base pairs.

3.3.5.3 Deletions

A total of 40 deletion mutations were recovered from the four E. coli strains which had been treated with 1,8-NONP (Table 15). Of these 40 deletions, the endpoints of 35 contain sequences exhibiting complete or partial (≥80 %) homology. In addition to those deletion mutations which have been sequenced, genetic and oligonucleotide probing studies provide evidence that at least 20 of the 38 mutants which were not recovered onto mRS81 contained class I or II deletions (section 3.3.4). The distribution of deletion mutations is given in

Table 15. LacI deletions recovered from E. coli strains treated with 1,8-NONP.

Site	# Occur.	Size	Endpoint Sequences 5'-flanking / deleted sequence / 3'-flanking	RB[a]
			NR6112 deletions	
-88-78	1	166	AGGCTATTC / TGGTGGCC...TGTCTCTTA / TCAGAC	4/5
147-269	3	122	AAGTGGAAG / CGGCGATGGC...GCAAATTGTCG / CGGCGATTAA	8
267-282	4	16	GCAAATTG / TCGCGGCGATTAAATC / TCGCGCCGATC	13/15
301-423	2	123	CAACTGGG / TGCCAGCGT...TGGATGACCAGGA / TGCCAT	7/8
306-316	1	11	GGGTGCCA / GCGTGGTGGTG / TCGATGGTAGAA	7/9
331-350	1	20	GTAGAACG / AAGCGGCGTCGAAGCCTGTA / AAGCGGCGGT	8
339-357	1	19	GAAGCGGCG / TCGAAGCCTGTAAAGCGGC / GGTGCACA	8
351-394	1	44	GAAGCCTGTAA / AGCGGCG...CAGTGGGCTG / ATCATTA	?
418-425	1	8	CTGGATGA / CCA GGATG / CCATTGCTG	8/9
454-796	1	343	CTGCACTAA / TGTTCCGGC...ATGCGCGCC / ATTACCG	4/5
572-874	1	303	ACCAGCAA / ATCGCGCCTGTTA...TGTTAT / ATCCCGCCGTTAAC	10/12
776-815	1	40	AGATG / GCGCTGGGCGCA...CCGGGCTGC / GCGTTGGTGCGG	9/11
788-796	1	9	GGGCGCA / ATGCGCGCC / ATTACCGAG	6/7
917-969	2	53	CCTGCTG / GGGCAAACCAG...GCGGTGAA / GGGCAATCAGCCTG	6
943-969	1	26	CCGCTTGCT / GCAACTCT...GCGGTGAA / GGGCAATCAGC	4
			EE125 deletions	
143-329	1	187	AAAAAGTG / GAAGCGGCGAT...GTAGAAC / GAAGCGGCGTC	9
250-353	2	104	CCTGCA / CGCGCCGTCGCAAATT...TAAAG / CGGCGGTGCACAAT	11/15
267-282	1	16	GCAAATTG / TCGCGGCGATTAAATC / TCGCGCCGATC	13/15
331-350	1	20	GTAGAACG / AAGCGGCGTCGAAGCCTGTA / AAGCGGCGGT	8
			NR6113 deletions	
-42-357	1	399	GACACCAT / CGAATGGTGCAA...AAAGCGGCG / GTGCACA	?
272-377	1	104	AAATTGTCGCG / CGCATTAAA...TCTTCTCGC / GCAACGCGT	8/10
			CM6114 deletions	
187-189	1	3	GCGTGGC / ACA / ACAACTGGC	5
267-282	4	16	GCAAATTG / TCGCGGCGATTAAATC / TCGCGCCGATC	13/15
339-357	1	19	GAAGCGGCG / TCGAAGCCTGTAAAGCGGC / GGTGCACA	8
828-837	1	11	GTTGGTGCGG / ATATCTCGGT / AGTGGCAT	4/5
917-969	1	53	CCTGCTG / GGGCAAACCAG...GCGGTGAA / GGGCAATCAGCCTG	6
929-943	2	15	GGGGCAAACCAGC / GTGGACCGCTTGCTG / CAACTCTCT	?
980-1022	1	43	GCAATCAG / CTGTTGCCC...TGGCGC / CCAATACGC	?

The sequence shown is that of the nontranscribed strand and is read 5' => 3'. The sequence is divided into three segments: the 5' flanking sequence; the deleted sequence; and the 3' flanking sequence. The convention for numbering deletions is to denote the first and last bases of the deleted sequence. The repeated bases at each endpoint are underlined.

[a] repeated bases at the endpoints

Table 16. Distribution of deletions in E. coli strains treated with 1,8-NONP.

Strain	NR6112 (wild-type)	EE125 (pKM101)	NR6113 (ΔuvrB)	CM6114 (pKM101, ΔuvrB)
sequenced deletions[a]	22	5	3	11
% sequenced mutants[b]	8.8%	3.9%	1.4%	5.0%
% nonhotspot mutants[c]	28%	7.4%	1.6%	5.2%
probable deletions[d]				
class I	1	2	2	1
class II	5	3	3	3
total deletions	28	10	7	15
% total mutants[e]	11%	6.7%	4.4%	6.2%

[a] Sequenced deletions have been characterized by DNA sequence analysis.
[b] the percentage of total characterized mutants which are deletions.
[c] the percentage of characterized nonhotspot mutations (i.e mutations other than +TGGC and -TGGC mutations) which are deletions.
[d] probable deletions have been partially characterized by genetic and oligonucleotide probing studies.
[e] deletion mutations which have been characterized completely (by DNA sequence analysis), or partially (by oligonucleotide probing and genetic methods) expressed as a percentage of the lacI⁻ mutants collected in each strain.

Table 16.

In spontaneous mutation spectra, deletions are the largest class of nonhotspot mutations, representing about 12 % of all mutants, and 40 % of nonhotspot mutants (Schaaper et al., 1986; Halliday and Glickman, unpublished results). In mutant collections derived from the four 1,8-NONP-treated E. coli strains, deletions represented 8.8 %, 3.9 %, 1.4 %, and 5.0 % of all characterized mutants in NR6112, EE125, NR6113, and CM6114 respectively, or 28 %, 7.4 %, 1.6 %, and 5.2 % of the nonhotspot mutations in these strains. When the putative deletions identified in genetic probing studies are included, deletions represented 10.6 % of the mutants selected from CM6112, 6.7 % of the EE125 mutants, 5.0 % of the CM6113 mutants, and 6.2 % of the mutants induced in CM6114.

3.3.5.4 Duplication, Complex, and Tandem Base Substitution Mutations

A small number of mutations which do not fall into categories discussed have been characterized by DNA sequencing. These are listed in Table 17.

A total of 30 mutations (5 from NR6112, 12 from EE125, 5 from NR6113, and 8 from CM6114 mutants) were recovered onto mRS81, but failed to yield any detectable change in sequence.

3.3.6 Summary of Mutational Spectra

A summary of the mutations collected from 1,8-NONP treated cultures of NR6112, EE125, NR6113, and CM6114 are given in Table 18. The results of all genetic, probing, and DNA sequence analyses for each nonhotspot mutant are provided in Appendix 1.

Table 17. Infrequent lacI⁻ mutations characterized by DNA sequence analysis.

a] Tandem base substitution, duplications, and complex mutation in NR6112

Mutant	Position	Mutation	Sequence Change[a]
K183	483/485	A(483) => G / -C (485)	TG ACC AGA => TG GC AGA
K158	189	Complex	GCAC AAC AACT => GCAC GCCC AACT
K248	65/66	GC => AA	GTAT GC CGGT => GTAT AA CGGT
K191	613-618	Duplication	GGCGCG TCTGCG TCTGG
K8	695-714	Duplication	GTGCC ATGTCCGGTTTTCAACAAAC CATGC

b] EE125 duplication

Mutant	Position	Mutation	Sequence change[a]
L70	613-618	Duplication	GGCGCG TCTGCG TCTGG

c] Double frameshift event and duplication recovered from in NR6113

Mutant	Position	Mutation	Sequence change[a]
M56	927/932	-G (927) / -G (932)	ACCA GCGTGG ACCG => ACCA CGTG ACCGC
M5	345-505	Duplication	CGTCGAAG CCTGTAAAG...ACAGTATT ATTTTTCTC

d] Tandem Base substitution, and duplication mutation recovered in CM6114

Mutant	Position	Mutation	Sequence Change[a]
N31	81/82	AG => TT	TTATC AG ACCG => TTATC TT ACCG
N165	863-883	Duplication	AGAC AGCTCATGTTATATCCCGCCG TTAA

[a] The DNA sequence of the mutated region is shown. The sequence that contains the changes is underlined. For duplications, the duplicated bases are underlined. The sequence shown is that of the nontranscribed strand.

Table 18. Summary of the distribution of lacI⁻ mutations in E. coli strains treated with 1,8-NONP.

	NR6112 (wild-type)	EE125 (pRM101)	NR6113 (ΔuvrB)	CM6114 (ΔuvrB,pRM101)
Mutation freq. (treated)	11.3	36	105	265
Mutation freq. (control)	5.4	7.3	3.2	3.0
Mutation freq. (X control)	2.1	5.0	33	88
Type of mutation				
Total +TGGC/-TGGC[a]	170 (63%)	61 (41%)	19 (12%)	6 (2.5%)
+TGGC	129	45	15	4
-TGGC	41	16	4	2
Base Substitutions				
transversions	6	35	5	76
G:C => T:A	5	31	4	66
G:C => C:G	1	1	1	2
A:T => T:A	0	1	0	7
A:T => C:G	0	2	0	1
transitions	5	4	7	4
G:C => A:T	5	3	7	4
A:T => G:C	0	1	0	0
Frameshifts				
-1	34	21	110	115
-2	3	0	2	3
+1	3	2	1	1
Deletions	22	5	3	11
Duplications	2	1	0	1
Tandems and complex	3	0	1	1
Total Characterized	248	129	148	218
Not Recovered	10	8	6	14
class I deletions[b]	1	2	2	1
class II deletion[b]	5	3	3	3
Not Characterized	5	12	5	8
Total mutants	263	149	159	240

a Spontaneous mutation hotspot: a frameshift mutation consisting of the addition or subtraction of one unit of a tandemly triplicated TGGC tetramer at position 621-632.

b Probable identity: assignment is based on genetic and oligonucleotide probing studies.

For comparative purposes, the most complete spontaneous spectra available for two of the DNA repair backgrounds (wild-type and pKM101) are given in Table 19. These spontaneous spectra are the work of Jennifer Halliday and Alasdair Gordon in Barry Glickman's laboratory at York University in Toronto.

3.3.7 Frequency and Induction of Mutational Events by 1,8-NONP

An estimate of the extent to which any single class of events (for example, frameshifts) was induced by 1,8-NONP can be made by comparing the spontaneous frequency of that event with its frequency following 1,8-NONP treatment (Table 20). This approach has limitations, the most notable of which is the tendency of PGal selection to overestimate the induced mutation frequency (see section 3.4.1.2). Nevertheless, the results are useful in identifying those classes of mutation which are most likely to have been induced by the chemical treatment.

The frequency of individual classes of mutation in 1,8-NONP treated E. coli strains (Table 20, column 1) may be calculated by considering the distribution of mutational events shown in Table 18, and the total mutational frequency. For +TGGC and -TGGC events and total characterized deletions, the frequencies have been calculated by considering the percentage of each event in the total mutant collection for each strain. For nonhotspot events that have been characterized by DNA sequence analysis, the frequency was estimated by considering the percentage of each event in the total number of characterized mutants. Mutational events such as duplications, tandem base substitutions, and complex mutations have not been

Table 19. Distribution of spontaneous mutants by class.

Genotype	wild-type	pKM101
Total +TGGC/-TGGC	70	61
+TGGC	56	45
-TGGC	14	18
Base substitutions	11	18
transversions	3.8	12
G:C => T:A	1.8	3.1
G:C => C:G	0.4	2.6
A:T => T:A	0.8	3.1
A:T => C:G	0.8	3.6
transitions	6.9	5.7
G:C => A:T	6.2	3.6
A:T => G:C	0.7	2.1
Frameshifts	5.0	5.7
-1	4.0	3.6
-2	0.1	1.0
+1/+2	1.0	1.0
Deletions	10	10
Addition/dup.	1.6	4.1
IS elements	1.0	0
Complex	0	0.5
Total # Mutants	726	193

Values given are percentages of the total number of lacI mutants examined. These data are unpublished results from J.Halliday and A. Gordon shown here with permission.

Table 20. Induction of mutational classes by 1,8-NONP.
The derivation of the frequencies of individual mutational classes in 1,8-NONP-treated, or untreated E. coli strains is described in the text.

[a] These values are calculated using the distributions and overall mutation frequencies for 1,8-NONP-treated cultures shown in Table 18.

[b] The total mutation frequency is for untreated (control) cultures as shown in Table 7. For NR6112 and NR6113, the distribution of spontaneous mutations in a wild-type background (Table 19) was used. The distribution of spontaneous mutants in a pKM101 background (Table 19) was used to estimate the frequency of individual mutational events in EE125 and CM6114. For CM6114, an alternate set of values were calculated (shown in parentheses) and are derived using the oligonucleotide probing results for hotspot events, and the pKM101 distribution for nonhotspot events.

[c] The values in this column are expressed as a "fold increase" over the spontaneous frequency (frequency in 1,8-NONP treated cultures/spontaneous frequency).

[d] Induced mutation frequency. The values in this column were calculated as (frequency in 1,8-NONP treated cultures - spontaneous frequency).

[e] Frequency of deletion mutations considering sequenced deletions only.

[f] Frequency of all deletion mutations: values include deletions characterized by genetic, oligonucleotide probing, or DNA sequencing studies.

Table 20. Induction of mutational classes by 1,8-NONP.

Strain	Mutation	Frequency ($\times 10^{-6}$) 1,8-NONP[a]	spontaneous[b]	fold increase[c]	IMF[d]
	Mut.freq.	11.3	5.4	2.1	5.9
NR6112 (wild-type)	+TGGC	5.5	3.0	1.8	2.5
	-TGGC	1.8	0.76	2.4	0.95
	Deletion[e]	1.0	0.54	1.8	0.45
	Deletion[f]	1.2	0.54	2.2	0.65
	Base Subst.	0.5	0.59	0.84	0
	Frameshift	1.8	0.27	6.7	1.5
	Mut.freq.	36.0	7.3	4.9	28.7
EE125 (pKM101)	+TGGC	10.8	3.3	3.2	7.5
	-TGGC	3.9	1.2	3.2	2.7
	Deletion[e]	1.4	0.73	1.9	0.67
	Deletion[f]	2.4	0.73	3.3	1.7
	Base Subst.	10.9	1.3	8.4	9.6
	Frameshift	6.4	0.42	15.2	6.0
	Mut.freq.	105	3.2	33	102
NR6113 (*uvrB*$^-$)	+TGGC	9.8	1.8	5.4	8.0
	-TGGC	2.6	0.45	5.8	2.2
	Deletion[e]	1.4	0.32	4.4	1.1
	Deletion[f]	4.4	0.32	13.8	4.1
	Base Subst.	8.4	0.35	24	8.0
	Frameshift	80.3	0.16	500	80
	Mut.freq.	265	3.0	88	262
CM6114 (*uvrB*$^-$, pKM101)	+TGGC	4.4	1.3 (0.43)	3.2 (10.2)	3.0 (4.0)
	-TGGC	2.2	0.48 (0.74)	4.6 (2.9)	1.7 (1.5)
	Deletion[e]	13.4	0.30 (0.47)	45 (28)	13 (13)
	Deletion[f]	16.5	0.30 (0.47)	55 (35)	16 (16)
	Base Subst.	97.2	0.54 (0.84)	180 (115)	97 (97)
	Frameshift	145	0.17 (0.26)	850 (560)	145 (145)

see footnote on opposite page.

included since too few of these were collected to establish their frequency.

The spontaneous frequencies for the major mutational classes are shown in the second column of Table 20. The distribution of mutational events used to obtain these values are those found in Table 19 for the wild-type and pKM101 DNA repair backgrounds. The distribution of spontaneous mutations in uvrB⁻ and pKM101, uvrB⁻ strains is not known with certainty, but can be estimated. If the assumption is made that the ΔuvrB mutation does not influence the distribution of spontaneous mutations (Schaaper et al., 1987), then the frequency of mutational classes in NR6113 (uvrB⁻) and CM6114 (pKM101, uvrB⁻) may be calculated using the spontaneous wild-type and pKM101 spectra (Table 19), respectively. However, since the distribution of hotspot mutations in spontaneous CM6114 mutants was different from the other 3 strains (see Table 8a), the frequency of mutational classes was also calculated using the following alternate assumptions: 1) that the distribution of +TGGC and -TGGC mutations in CM6114 can be estimated from table 8a; and 2) that the distribution of spontaneous nonhotspot events is similar to that in the spontaneous pKM101 spectrum. Estimates of the spontaneous mutation frequency calculated in this fashion are shown in parentheses in Table 20 (column 2). It should be emphasized that since the induced mutation frequency is relatively high in strains NR6113 and CM6114, the majority of the mutations recovered are likely to have been induced by 1,8-NONP. Therefore errors that are made in estimating the spontaneous frequency of mutational events in these

strains have little influence on the final results.

Direct comparison of the values in the first 2 columns of table 20 permits us to address the question of the mutational events that are induced in the presence of the mutagen. The outcome can be expressed either as a "fold increase" over control (column 3) or as an induced mutation frequency (column 4). The latter column provides an estimate of the induced mutation spectrum.

In NR6112 frameshift mutations were increased markedly (6.7-fold) and comprised about 30 % of the induced mutation spectrum. This compares to the 4 % contribution that frameshifts make to the spontaneous spectrum in this strain. While the frequency of other mutational classes (+TGGC, -TGGC, and deletions) was increased slightly above spontaneous levels, it is likely that a significant proportion of these mutations are of spontaneous origin. 1,8-NONP treatment of EE125 resulted in only a slight increase in +TGGC (3.2X), -TGGC (3.2X), sequenced deletion (1.9X), and total deletion mutations (3.3X). However, both base substitutions (8.4X) and frameshifts (15.2X) were increased considerably and represented 37 % and 22 % of the induced spectrum respectively.

All classes of mutation appear to have been induced by 1,8-NONP in the two strains which were deficient in excision repair. In NR6113 frameshift mutations clearly predominated, comprising 80 % of the induced mutation spectrum. Base substitutions (8 %), deletions (2 %), and +/-TGGC mutations (10 %) constituted the remaining portion of the induced spectrum in this strain. In CM6114, the distribution of events as a percent of induced mutations was: frameshifts 55 %,

base substitutions 37 %, deletions 5 %, and hotspot mutations 2 %.

The results shown in Table 20 suggest that the classes of mutation most affected by 1,8-NONP treatment were frameshift and base substitution mutations. The predominant feature in all strains was the degree to which frameshift mutations were induced. This is particularly the case in strains deficient in excision repair. While 1,8-NONP increased the frequency of frameshift mutations by 6.7-fold in NR6112 and 15.2-fold in EE125, identical treatment of the uvrB$^-$ derivatives NR6113, and CM6114 produced an elevation in frameshift mutation of 500-fold and 560-850-fold, respectively. The frequency of base substitution mutations was increased considerably in E. coli strains harboring the plasmid pKM101. Taking the values from Table 20 (column 4) it can be seen that the ratio of induced frameshifts to induced base substitutions in strains containing pKM101 was 0.6 : 1 and 1.5 :1 in strains EE125 and CM6114, respectively. In contrast, the induced frameshift : base substitution ratio in NR6113 was 10 : 1.

The frequency of some mutational classes appear to be only slightly increased by 1,8-NONP, and therefore it is more difficult to determine whether these mutations were induced by the treatment or were of spontaneous origin. Deletions were induced considerably in CM6114, but much less in NR6112, EE125 or NR6113. Mutations at the +/-TGGC hotspot also appear to have been induced slightly in all E. coli strains. However, in relative terms these mutations make a minor contribution to the induced mutation spectrum.

3.3.8 The Specificity of Frameshift Mutations Recovered Following 1,8-NONP Treatment

Frameshifts were induced by 1,8-NONP more extensively than any other mutational class in all E. coli strains studied. This result was expected on the basis of the extraordinary ability of 1,8-DNP to induce frameshift mutations in S. typhimurium strains TA1537, TA1538, and TA98 (Table 1; Rosenkranz and Mermelstein, 1983; Tokiwa and Ohnishi, 1986) which often revert as the result of frameshift mutations at G:C base pairs (Hartman et al., 1986). In the present study, the majority of frameshift mutations occurred at G:C base pairs (85 % in NR6112, 87 % in EE125, and >99 % in both NR6113 and CM6114).

Most frameshift mutations involve the loss of one base in a run of G or C. The dependence of mutation on the length of the reiterated sequence was examined by expressing the number of -1 frameshift events recovered at guanine runs as a function of the target size (S) (S = length of the run (R) multiplied by the number of detectable sites of size R in the lacI gene). This is shown in Table 21. To obtain S, only runs of G (or C) prior to, or at, position 1012 were considered since frameshifts sites after this point have never been observed (Calos and Miller, 1981) and are probably phenotypically silent (see section 3.4.1.1). Figure 22 shows a plot of frameshift events as a function of the length of the guanine run and shows that the frequency of frameshift mutations increases markedly with the length of the reiterated sequence.

The size of the collection of sequenced frameshift mutations

Table 21. The dependence of frameshift mutagenesis on the length of the reiterated guanine sequence.

Strain			NR6112 (wild-type)	EE125 (pKM101)	NR6113 (ΔuvrB)	CM6114 (pKM101, ΔuvrB)	
Length[a]	# sites[b]	target[c]					weighted average[d]
1	312	312	-	0.035[e]	0.018	0.011	0.014
2	81	162	0.15	0.10	0.16	0.13	0.14
3	23	69	0.95	1.0	0.80	0.94	0.89
4	1	4	2.6	-	3.2	2.6	2.7

[a] The number of G:C base pairs in the reiterated sequence.
[b] The number of sites with the specified G:C sequence which occur at or prior to position 1012.
[c] The target size is defined here as the total number of G:C base pairs within contiguous sequences of a specified length at sites which yield the i^- phenotype when a -1 frameshift occurs (this is equal to length multiplied by the number of sites).
[d] The weighted average is calculated by considering the total number of mutants at sites of the specified length, and then normalizing the value to a hypothetical population of 100 frameshift mutants. Therefore the strains with the largest number of frameshift mutations contribute most to this weighted average.
[e] Values are expressed as # occurrences/target size for a hypothetical population of 100 frameshift mutants.

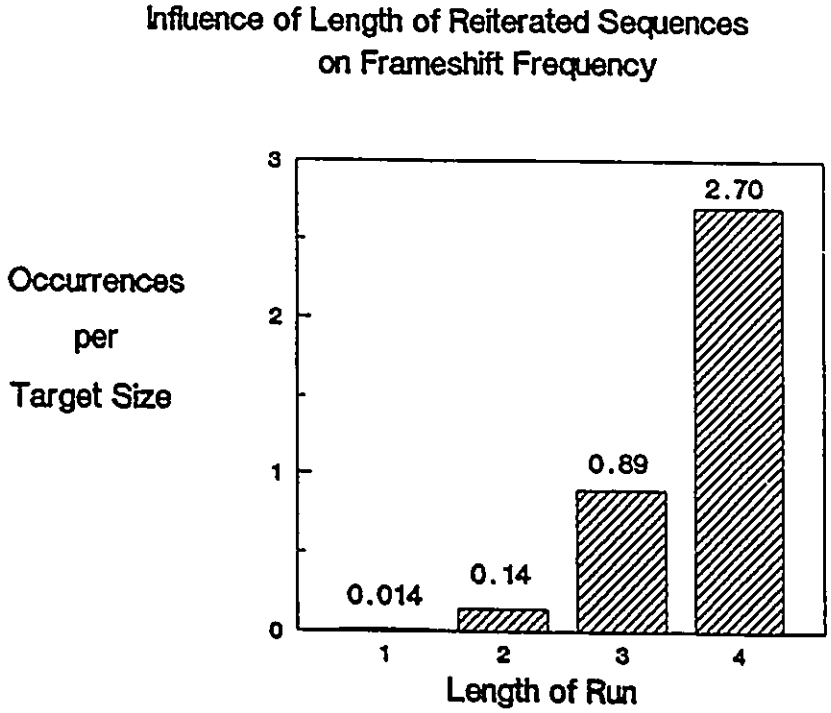


Figure 22. Influence of reiterated guanine sequences on frameshift frequency.
The values plotted are the number of frameshift mutations detected (as a function of the target size) for a hypothetical population of 100 frameshift mutants versus the number of guanine residues in the reiterated sequence. The "occurences per target size" is a weighted average for all strains, but the general trend is similar for each individual strain (see Table 21).

makes it possible to pose questions regarding the influence of neighboring bases on the mutability of particular sites. To facilitate this analysis, the detectable frameshift sites in the lacI gene, and the characterized mutations were classified according to their flanking bases as shown in Table 22. The influence of the 5'- or 3'-flanking base on -1 frameshift occurrence was analyzed statistically using the Chi-square goodness of fit test. When statistical analysis was applied to frameshift mutations recovered from each E. coli strain, individual cell counts were often below the number of occurrences which yield accurate results in this test (Malik and Mullen, 1973; Sokal and Rohlf, 1981). Therefore, values for all strains were pooled as shown in Table 23.

A total of 65 frameshift mutations occurred at runs of 2 guanines (NGGN sites). Seventy-eight percent of these mutations occurred at sites which have a 5'-flanking T (TGGN): 85 % in NR6112, 79 % in NR6113, 78 % in CM6114, and 67 % in EE125. Only 53 % of detectable NGGN frameshift sites contain a 5'-flanking T. The bias towards -1 frameshifts at TGGN sites is statistically significant ($P<0.001$). When the base at the 3'-end of NGGN frameshift sites was examined, it was found that 53 % of the mutations recovered following 1,8-NONP treatment occurred at sites with a 3'-flanking adenine (NGGA), although only 20 % of the detectable sites have this base at the 3'-end. The tendency for mutations to occur at NGGA sites is also significant ($P<0.001$).

One hundred and sixty-six frameshift mutants occurred at sites containing 3 contiguous guanines (NGGGN sites). When the

Table 22. Distribution of -(G:C) frameshift mutations.

Strain		NR6112 (wild-type)	EE125 (pKM101)	NR6113 (ΔuvrB)	CM6114 (pKM101, ΔuvrB)	
sequence context	# sites					Total
AGA	29	0	0	0	0	0
AGT	21	0	0	0	0	0
AGC	35	0	1	0	0	1
CGC	67	0	0	0	0	0
CGA	32	0	0	3	0	3
CGT	33	0	0	0	0	0
TGT	24	0	0	0	1	1
TGA	28	0	1	1	1	3
TGC	43	0	0	2	4	6
AGGA	2	0	0	3	2	5
AGGC	6	0	0	0	1	1
AGGT	1	0	0	0	0	0
CGGC	11	0	0	2	1	3
CGGA	7	0	0	1	1	2
CGGT	11	1	1	1	0	3
TGGT	18	1	0	0	5	6
TGGA	7	1	0	21	6	28
TGGC	18	4	2	5	6	17
AGGGA	0	-	-	-	-	-
AGGGT	0	-	-	-	-	-
AGGGC	4	1	1	13	10	25
CGGGC	4	4	0	12	4	20
CGGGA	4	1	5	16	23	45
CGGGT	0	-	-	-	-	-
TGGGT	3	2	1	8	9	20
TGGGA	4	8	3	3	16	30
TGGGC	4	3	3	8	12	26
TGGGGC	1	3	0	14	12	29
Totals		30	18	110	114	272

This table shows the distribution of -1 frameshift mutations recovered according to the sequence flanking the repeat from which the G is deleted. The values given are the number of mutations which occurred at each type of sequence. The deleted guanine is one of the underlined residues.

Table 23. Analysis of the distribution of -1 frameshift mutations recovered following 1,8-NONP treatment

Strain		NR6112 (wild-type)	EE125 (pKM101)	NR6113 (ΔuvrB)	CM6114 (pKM101, Δ uvrB)	Total		
Context[a]	#sites					o	e	$(o-e)^2/e$
AGGN	9	0	0	3	3	6	7.2	0.2
CGGN	29	1	1	4	2	8	23.2	9.95
TGGN	43	6	2	26	17	51	34.5	7.88
Totals						65 different	65	X^2=18.03 (P<0.001)
AGGGN	4	1	1	13	10	25	28.8	0.50
CGGGN	8	5	5	28	27	65	57.7	0.92
TGGGN	11	13	7	19	37	76	79.4	0.14
Totals						166 no difference	166	X^2=1.56
NGGA	16	1	0	25	9	35	12.8	38.3
NGGC	35	4	2	7	8	21	28	1.79
NGGT	30	2	1	1	5	9	24.1	9.42
Totals						65 different	65	X^2= 49.5 (P<0.001)
NGGGA	8	9	8	19	39	75	57.7	5.16
NGGGC	12	8	4	33	26	71	86.6	2.81
NGGGT	3	2	1	8	9	20	21.6	0.12
Totals						166 different	166	8.09 (P<0.025)

The statistical analysis has been done by chi-square analysis for goodness of fit. The effect of the flanking base (5' or 3') on the total collection of frameshift mutations recovered following 1,8-NONP treatment.

[a] The context of the contiguous G's refers to the bases 5' and 3' to the repeat: N = any base; N = any base.

o: observed distribution of frameshift mutations in each strain according to the nature of the flanking base.

e: expected distribution of frameshift mutations as calculated by the number of detectable sites with the specified flanking bases.

X^2: the chi-square statistic which is equal to $(o-e)^2/e$. The significance of the statistic is evaluated at 2 degrees of freedom.

nature of the 5'-flanking base at these sites was considered, it was found that the distribution of frameshift mutations recovered from 1,8-NONP treated strains was not significantly different from the distribution of detectable NGGGN sites. As with frameshift mutations at NGGN sites, it was found that NGGGN sites containing a 3'-flanking A were more highly represented in the 1,8-NONP-induced mutation spectrum (45 %) than was expected on the basis of the number of detectable NGGGN sites containing this base (35 %). While this difference is statistically significant ($P<0.025$), the effect of the 3'-flanking adenine at NGGGN sites was not as marked as at NGGN sites. Too few frameshift mutations occurred at sites containing a lone guanine residue (NGN sites) to justify statistical analysis. However, 10 out of 14 (71 %) of the frameshifts at NGN sites had a 5'-flanking T compared to 30 % of the detectable sites. Similarly, 7 out of 14 (50 % of the NGN frameshifts occurred at sites with a 3'-flanking A (compared to 28% of the detectable NGN sites).

Of the 295 frameshift mutations recovered following 1,8-NONP treatment, the vast majority (288) were due to the loss of bases. Only 7 (2.4 %) of the frameshift mutations sequenced resulted from the addition of bases. When only G:C base pairs were considered, the proportion of additions was even smaller (about 1 %). This is in contrast to spontaneous frameshift mutations in the lacI gene (Table 19), or frameshifts induced by the intercalating agent ICR-191 (Calos and Miller, 1981) which are the result of base additions as well as base losses. This suggests that 1,8-NONP acts by a mechanism which preferentially induces the loss of bases.

The analysis of sequences at which frameshifts occur suggests that these mutations exhibit considerable specificity which can be summarized as follows: 1) most frameshifts occur at G:C sites; 2) most events occur at sequences of contiguous guanines; 3) The probability of a frameshift occurring increases markedly with the length of the reiterated sequence; 4) frameshift events at NGN or NGGN sites are highly favoured when the 5'-flanking base is thymine and/or the 3'-flanking base is adenine; 5) frameshift events at NGGGN sites are favoured at sites which contain a 3'-flanking adenine; and 6) losses of G:C base pairs are much more prominent than the addition of bases. Possible mechanisms which are consistent with the specificity observed for 1,8-NONP-induced frameshift mutation will be considered in the Discussion.

A total of 13 frameshift mutations characterized in these studies involve the gain (4) or loss (9) of A:T base pairs. The majority of these (12) occur at runs of contiguous adenines ranging from 2 to 5 bases. Frameshifts characterized at A:T base pairs following 1,8-NONP treatment differ from frameshifts induced at G:C base pairs in at least 2 respects: the percentage of base additions is much higher at A:T sites (31 %) than G:C sites (1 %); and a greater number of the frameshifts involving A:T base pairs occur in *uvrB*$^+$ strains (which have relatively low induced mutational frequencies) than in *uvrB*$^-$ strains. This suggests that some of these might be of spontaneous origin.

3.3.9 The Specificity of Base Substitution Mutations Recovered Following 1,8-NONP Treatment

Base substitution mutations comprised a very minor proportion of the mutational spectrum in strains that lacked plasmid pKM101 (ie. strains NR6112 and NR6113). However in the pKM101-containing strains EE125 and CM6114, the incidence of base substitution mutations increased markedly. As with frameshift mutations, the vast majority of these occurred at G:C base pairs (11/11 in NR6112, 12/12 in NR6113, 35/39 in EE125, and 72/80 in CM6114).

The types of base substitution recovered were markedly dependent on the presence of plasmid pKM101. In strains which lacked pKM101 the ratio of transversions to transitions was about 1:1 whereas in the two strains which contained pKM101, the majority of base substitutions were transversions, specifically G:C => T:A changes. A total of 106 G:C => T:A transversions (out of 142 total base substitution mutations) were recovered at 55 different sites in the lacI gene. Forty-four of these were nonsense mutations and 62 were missense mutations.

An analysis of the site specificity of base substitution mutations is not so straightforward as for frameshift mutations. Unlike frameshift mutations which, to a first approximation, can be expected to inactivate the repressor to the same extent at all detectable sites, base substitution mutations will produce a range of altered lactose repressor molecules. Some of these will be normal enough to be phenotypically silent while others will be defective enough to be selected by the methods employed. Still others might be

expected to produce a partially active protein. The amber/ochre nonsense mutation system developed by Miller and his colleagues (Miller et al., 1977; Schmeissner et al., 1977a; Coulondre and Miller, 1977a) circumvented this problem by analyzing only nonsense mutations, which inactivate the repressor to approximately the same extent at each detectable site. However, the nature of the DNA sequences which surround different nonsense sites are extremely limited. For instance, there are 26 nonsense sites in the lacI gene at which G:C => T:A transversions can be detected: at 19 of these sites guanine is in a GGA, CGA, or TGA context, and fully 50 % of the sequence contexts in which guanine could theoretically be situated are not represented at all in the nonsense system (Table 24). The DNA sequencing system used in these studies allows the analysis of both nonsense and missense mutations. There are a total of 409 guanines which, when altered by a G:C => T:A transversion, give rise to an altered protein (i.e. which result in an amino acid change). This total does not include sites after nucleotide 1013 (no missense or nonsense mutations have ever been characterized after this site), or sites at which base substitution does not alter the codon message. Of these 409 potential sites, there are 75 known G:C => T:A transversion sites. These 75 sites include 26 nonsense sites described by Miller et al. (1978), 36 missense sites described by Gordon et al. (1988a), 10 previously unreported sites documented in the data in Table 11 and Appendix 2, and another 3 sites recently found in the laboratory of Barry Glickman (personal communication). Although it is likely that there are other sites which might produce

Table 24. The distribution of G:C => T:A transversions according to the nature of the bases flanking the mutated G.

a) Total G:C => T:A transversions

Strain		NR6112	EE125	NR6113	CH6114	Total
Context	# Sites					
[illegible]	6	0	2	1	3	6
[illegible]	9	1	3	0	7	11
[illegible]	4	1	0	1	0	2
[illegible]	9	0	5	0	9	14
[illegible]	1	0	0	0	2	2
[illegible]	9	1	3	0	14	18
[illegible]	6	0	2	1	3	6
[illegible]	8	0	5	0	5	11
[illegible]	2	0	0	0	3	3
[illegible]	6	1	4	0	9	13
[illegible]	2	0	2	0	1	3
[illegible]	4	1	2	0	4	7
[illegible]	0	0	0	0	0	0
[illegible]	7	0	3	0	6	9
[illegible]	1	0	0	1	0	1
[illegible]	1	0	0	0	0	0
Totals	75	5	31	4	66	106

b) G:C => T:A transversions which are nonsense mutations

Strain		NR6112	EE125	NR6113	CH6114	Total
Context	# Sites					
[illegible]	1	0	2	0	0	2
[illegible]	7	1	2	0	5	8
[illegible]	0	0	0	0	0	0
[illegible]	0	0	0	0	0	0
[illegible]	0	0	0	0	0	0
[illegible]	8	1	3	0	14	18
[illegible]	2	0	1	0	0	1
[illegible]	0	0	0	0	0	0
[illegible]	0	0	0	0	0	0
[illegible]	4	1	4	0	6	11
[illegible]	1	0	0	0	1	1
[illegible]	1	0	0	0	0	0
[illegible]	0	0	0	0	0	0
[illegible]	2	0	1	0	2	3
[illegible]	0	0	0	0	0	0
[illegible]	0	0	0	0	0	0
Totals	26	3	13	0	28	44

c) G:C => T:A transversions which are missense mutations

Strain		NR6112	EE125	NR6113	CH6114	Total
Context	# Sites					
[illegible]	5	0	0	1	3	4
[illegible]	2	0	1	0	2	3
[illegible]	4	1	0	1	0	2
[illegible]	9	0	5	0	9	14
[illegible]	1	0	0	0	2	2
[illegible]	1	0	0	0	0	0
[illegible]	4	0	1	1	3	5
[illegible]	8	0	5	0	5	10
[illegible]	2	0	0	0	3	3
[illegible]	2	0	0	0	3	3
[illegible]	1	0	2	0	0	2
[illegible]	3	1	2	0	4	7
[illegible]	0	0	0	0	0	0
[illegible]	5	0	2	0	4	6
[illegible]	1	0	0	1	0	1
[illegible]	1	0	0	0	0	0
Totals	49	2	18	4	38	62

a phenotypically selectable mutant repressor when mutated by G:C => T:A transversions, it will be assumed in the following analysis that these 75 sites give a reasonable approximation of the total lacI G:C => T:A target.

The influence of flanking bases on the mutability of particular guanine sites may be examined by comparing the observed distribution of independent mutations to the distribution of the detectable sites. The distribution of both the 1,8-NONP-induced G:C => T:A transversions, and the potential sites of mutation is shown in Table 24. It should be noted that the total detected G:C => T:A transversion sites are more uniformly distributed than either the nonsense sites or missense sites when classified according to the nature of the nucleotides flanking the guanine. Comparison of the expected and observed distribution of transversions was done using the Chi-square goodness of fit test as shown in Table 25. The analysis was confined to the pKM101 containing strains EE125 and CM6114 since only a small number of G:C => T:A transversions were found in collections of mutants isolated from the other two strains. Both the 5' and 3' flanking bases were tested in independent analyses. The distribution of G:C => T:A transversions recovered from EE125 which had been treated with 1,8-NONP was not significantly different from the expected distribution. In the collection of transversions recovered from CM6114 there appeared to be a slight bias for sites that contained a 3'-purine or a 5'-pyrimidine, but neither of these factors showed a statistically significant departure from what was expected. This analysis suggests that, for 1,8-NONP-

Table 25. Analysis of the distribution of G:C => T:A transversions recovered following 1,8-NONP treatment.

Strain		EE125 (pKM101)			CM6114 (pKM101, Δ uvrB)		
a] Effect of 3' base							
Context[a]	#sites	o	e	$(o-e)^2/e$	o	e	$(o-e)^2/e$
NGG	9	2	3.72	0.795	8	7.92	0.00
NGA	31	13	12.8	0.002	36	27.28	2.78
NGT	13	4	5.37	0.35	4	11.44	4.84
NGC	22	12	9.09	0.93	18	19.36	0.01
Total	75	31	31	X^2=2.08	66	67	X^2=7.63
		no difference P>0.5			no difference P>0.05		
b] effect of 5'base							
Context[a]	#sites	o	e	$(o-e)^2/e$	o	e	$(o-e)^2/e$
GGN	28	10	11.57	0.21	19	24.64	1.29
AGN	9	3	3.72	0.14	6	7.92	0.46
TGN	14	8	5.79	0.85	17	12.32	1.78
CGN	24	10	9.92	0.001	24	21.12	0.39
Total	75	31	31	X^2=1.20	66	66	X^2=3.92
		no difference P>0.75			no difference P>0.25		

The statistical analysis has been done by chi-square analysis for goodness of fit. The effect of the flanking base (5' or 3') on the total number of G:C => T:A transversions collected in EE125 and CM6114 was examined. Insufficient numbers of base substitutions were recovered in the other strains to warrant consideration.

[a] The context of the mutated G refers to the bases 5' and 3' to the base: N = any base.

o: observed distribution of base substitution mutations in each strain according to the nature of the flanking bases.

e: expected distribution of base substitution mutations as calculated by the number of detectable sites with the specified flanking bases.

X^2: the chi-square statistic which is equal to $(o-e)^2/e$. The significance of the statistic was evaluated at 3 degrees of freedom.

induced base substitutions, the bases flanking a guanine do not exert a significant influence on the mutability of the site.

In CM6114, 8 base substitution mutations occurred at A:T base pairs. Seven of these were A:T => T:A transversions which occurred at 7 different sites; one A:T => C:G transversion was also recovered. It is striking that of the 80 base substitution mutations collected from CM6114, 73 were transversions that would arise by the misincorporation of adenine across from a purine during DNA replication (66 G:C => T:A; 7 A:T => T:A). Four base substitutions in EE125 occurred at A:T base pairs. Although the numbers are small, the specificity is clearly different (1 A:T => G:C transition, 2 A:T => C:G transversions, and 1 A:T => T:A transversion) from the A:T base substitutions isolated from CM6114.

3.4 DISCUSSION

3.4.1 Analysis of the Mutational Specificity of 1,8-NONP in the lacI Gene: Advantages and Limitations

3.4.1.1 The Mutational Target

One of the most attractive features of the lacI system used in the experiments described here is the large size of the mutational target. The target sequences in most other systems generally range from fewer than 100 base pairs to about 250 base pairs, while the mutational target in the lacI gene is about 1000 base pairs (see below). The diversity of the DNA sequence in such a large target allows us to determine whether sequences which flank mutational sites influence the distribution of mutation. However, it should be recognized that the monitorable target (i.e. the sequences which, when altered, yield a selectable phenotype) might differ for each class of mutation. This can have a significant influence on: 1) the relative proportions of different types of mutations which are recovered; and 2) the observed distribution of those mutations. Fortunately, the lacI gene has been studied extensively in several laboratories; on the basis of those studies it is possible to estimate the size of the monitorable target for different classes of mutation.

a) Point Mutations. Mutations have been recovered frequently in approximately 1000 base pairs of the lacI gene. A variety of evidence suggests that frameshifts and base substitutions beyond position 1013 will not produce an i^- phenotype which can be selected on PGal. 1) The two most comprehensive studies of frameshift

mutation which have been done in the lacI gene are the study by Calos and Miller (1981) in which 365 ICR-191-induced frameshift mutations were characterized by a combination of genetic and DNA sequencing methods, and this study (295 1,8-NONP-induced frameshifts). In the ICR-191 study the frameshift mutation nearest the 3'-end of the lacI gene was at position 986-988, while in the present experiments frameshifts were not detected beyond position 1011-1012. Both studies recovered mutations at each of the 23 NGGGN sites up to and including the one at position 986-988. Since a potential NGGGN site occurs at position 1014-1016, but was not detected in either study, it is likely that frameshifts at this site do not yield a selectable phenotype. 2) No nonsense mutations have been detected after codon 314 (position 1007-1009) (Miller et al., 1978). 3) A threonine to proline change caused by a A:T => C:G transversion at position 1013 was the missense mutation site closest to the 3'-end documented in a review of lacI missense mutations (Gordon et al., 1988a). In light of this evidence, it can be estimated that the monitorable target for point mutations extends from about position 29 (start of initiation codon) to 1013.

Within the monitorable target, virtually all frameshift and nonsense mutations produce a defective repressor and can be selected on PGal. However, only a fraction of mutants which contain missense mutations are expected to form colonies on selective media (see section 3.3.9). Gordon et al. (1988a) have analyzed the distribution of missense mutations which have been detected in the lacI gene. They noted that the missense sites were clustered in regions of the

gene encoding information essential to repressor function: over one half of the detected mutations yielding amino acid replacements occurred in the initial 180 base pairs of the gene which encode the DNA binding domain; the portion of the gene encoding the protein core was relatively insensitive to missense mutation. Miller et al. (1979) determined the location of lacI missense mutations by means of deletion mapping, and also noted extensive clustering of amino acid replacements which yielded i⁻ cells. The present study provides additional evidence that detectable missense mutations are clustered in lacI sequences which encode amino acids essential to repressor function. Of the 142 base substitution mutations which were recovered following 1,8-NONP treatment, 90 were missense mutations and 52 were nonsense mutations. Fifty-six of the missense mutations (36 different substitutions) occurred in the initial 180 base pairs; the remaining 34 missense mutations (23 different substitutions) occurred in the remaining 800 base pairs of the target sequence. In contrast, nonsense mutations and frameshift mutations were much more evenly distributed along the lacI gene. Therefore, relative to other classes of mutation, the total number of base substitutions (nonsense and missense) which occur in both treated and untreated cells is probably greater than is estimated by lacI⁻ spectra.

b) Deletions and Duplications/Additions. The target for mutations which arise as the result of the loss or gain of several bases is less well defined. It is likely that large deletions of several kilobases extending into the lacI gene from the 5'-end, deletions whose endpoints are within the lacI gene, and deletions

which extend from the lacI gene into the lac operator will yield a selectable phenotype. Most mutations resulting from the gain of bases (duplications, additions, or insertion elements) within the lacI gene are also expected to destroy repressor function. The major limitation for characterization of such mutations is likely to occur at the cloning step. In some cases it is likely that, due to extensive damage of the gene, the frequency of recombination onto the M13 vector will be too low to permit recovery. Deletion mapping and oligonucleotide probing studies (Table 10) suggest that at least 20 of the 38 mutations which were not recovered into mRS81 were deletions. The largest deletion recovered in this study was 399 base pairs in length, and the largest duplication was 160 base pairs. Large mutations reported to have been recovered into mRS81 include a deletion of 789 base pairs (Fix et al., 1987), a duplication of 457 base pairs, (Schaaper et al., 1986), and an insertion (IS1) of 768 base pairs (Schaaper et al., 1986; 1987; Fix et al., 1987).

3.4.1.2 Selection of lacI$^-$ Mutants

The use of a F'lac which contains the I^q and I8 promotor mutations is advantageous because the majority of colonies which grow on PGal contain a lacI$^-$ mutation (section 3.1.1.1) (Miller et al., 1977; Miller, 1978). A disadvantage of this method, as discussed above, is that the selection is somewhat biased towards mutations which destroy repressor function.

The calculations of mutation induction (Table 20) clearly show that some mutational classes (such as frameshifts and base substitutions) were effectively induced by 1,8-NONP. However, taken

at face value, they also suggest that +TGGC and -TGGC mutations were induced by about 2 to 3-fold in strains which are uvrB$^+$, and that these spontaneous hotspot mutations comprised almost 60% of the 1,8-NONP-induced NR6112 spectrum and 36% of the induced EE125 spectrum. Although it is possible that 1,8-NONP specifically induced +TGGC and -TGGC mutations (see section 3.4.5), an alternative, and more likely, possibility is that the overall mutation frequency of 1,8-NONP-treated cultures was overestimated. A review of the selection protocol suggests a possible reason for overestimation of the induced mutation frequencies. In order to calculate mutation frequency, the total number of cells plated onto PGal was estimated by plating aliquots of the cultures onto nonselective medium (LB) (section 3.2.2.1). However, agar plates containing PGal inevitably contain residual amounts of alternative carbon sources which can sustain a limited amount of growth on the plate; the extent of individual cell growth is dependent on the cell density (Smith and Sadler, 1971; Miller, 1972). In order to count the larger number of lacI$^-$ mutants which were formed in 1,8-NONP treated cultures, it was necessary to plate a smaller number of treated than untreated bacteria onto PGal, allowing treated cells a relatively longer period of growth on the plate. This would lead to an overestimation of the mutational frequency in cultures plated at low cell density (ie. 1,8-NONP-treated cultures) relative to high cell density (untreated cultures). In order to ensure that selection conditions following 1,8-NONP treatment were identical for all 4 E. coli strains, approximately the same number of cells of each treated culture were plated onto PGal

plates. Since survival is about the same (Table 7) in all of the treated strains, any overestimation would be similar in degree. On the basis of the induction of +TGGC and -TGGC events in the uvrB$^+$ strains (which were not mutated extensively by 1,8-NONP), it is probable that increases of 2 to 3-fold are within the limit of uncertainty for estimation of the mutational frequency.

3.4.2 Base Substitution Mutations

During replication of the bacterial genome, fidelity is ensured by three major processes: 1) the specificity of the polymerase for the correct base; 2) 3'=> 5' exonucleolytic removal of incorrect bases by the proofreading activity associated with DNA polymerase; and 3) postreplicational recognition and removal of incorrect bases from the daughter strand by mismatch repair enzymes (Loeb and Kunkel, 1982). Historically, consideration of the mechanisms by which bulky adducts induce base substitution mutations has emphasized the first of these steps: i.e. how the wrong base(s) might be misincorporated opposite the lesion. In the following discussion a similar approach is taken. It should be noted however, that the final distribution of mutations could be influenced considerably at steps 2 and 3 if there were differential repair of various types of adduct:base mispairing. It is known that in unmodified DNA, the efficiency with which different mismatches are corrected by the proofreading activity of DNA polymerase III can differ by two orders of magnitude (Fersht et al., 1982; Fersht and Knill-Jones, 1983), and that the mismatch enzymes of E. coli do not repair all mismatches equally (Dohet et al., 1985). Whether these

observations are also relevant to mismatches involving adducts is unknown.

3.4.2.1 G:C => T:A Transversions

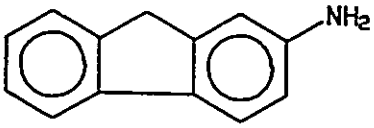
Base substitution mutations represented a major portion of the 1,8-NONP-induced mutational spectrum in both of the E. coli strains (EE125 and CM6114) which contained the plasmid pKM101. The base substitutions had the following specificity: 1) ninety percent occurred at G:C base pairs; 2) virtually all (>93 %) were transversions, of which G:C => T:A changes predominated; and 3) the nature of the 3'- and 5'-flanking bases did not appear to influence the sites at which these G:C => T:A transversions occurred.

There have been no previous studies which have determined the DNA sequence of base substitution mutations induced in bacteria by 1,8-NONP or 1,8-DNP. However, results from studies which have examined the mutagenicity of 1,8-DNP using reversion assays are consistent with the results obtained here. Using the trpA reversion system, McCoy et al (1985) showed that 1,8-DNP induced base substitutions in E. coli strains which were deficient in excision repair and contained the plasmid pKM101. Reversion to tryptophan prototrophy was not detected in isogenic strains lacking plasmid pKM101. Similarly, studies using the S. typhimurium tester strains developed by Ames and coworkers (Maron and Ames, 1983) established that 1,8-DNP induced base substitutions at the hisG46 locus in strains which contained the plasmid pKM101 (i.e in TA100 but not in TA1535) (Table 1; Rosenkranz and Mermelstein, 1983; Tokiwa and Ohnishi, 1986). The hisG46 locus reverts as a result of G:C => T:A

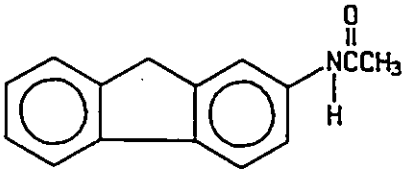
and G:C => C:G transversions, and G:C => A:T transitions at a target sequence of 3 consecutive G:C base pairs (Hartman et al., 1986). However, revertants in hisG46 strains can also appear as the result of suppressor mutations which are the result of an A:T => C:G transversion in a tRNA gene (Levin and Ames, 1986). Therefore, in order to obtain useful information regarding mutagenic specificity, revertants must be further characterized at the DNA sequence level (Miller and Barnes, 1986; Eisenstadt et al., 1989). On the basis of the results presented here, it can be predicted that 1,8-NONP or 1,8-DNP-induced reversion of TA100 is largely a result of G:C => T:A transversions at the hisG46 mutation.

Mutations arising at sites of DNA damage are referred to as targeted mutations. The fact that the majority of base substitution mutations occurred at G:C base pairs suggests that they were targeted by an adduct formed with guanine or cytosine. In chapter 2, evidence was presented that the major adduct (about 95 % of the total DNA adducts) formed by 1,8-NONP in E. coli is dG-C(8)-ANP and it is probable, though not certain, that this adduct is the premutational lesion. The only other adduct that was formed with guanine or cytosine was an unidentified minor product which accounted for about 2.5 % of the total DNA adduct (Figure 8).

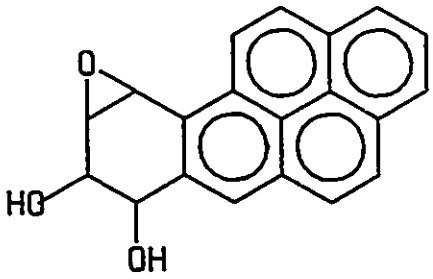
A review of the specificity of base substitutions induced by other mutagens which react with guanine (Figure 23) shows that G:C => T:A changes are common. Like 1,8-NONP, 2-aminofluorene (AF) and 2-acetylaminofluorene (AAF) react with the C(8) position of guanine. In the tetracycline resistance gene of plasmid pBR322, the base



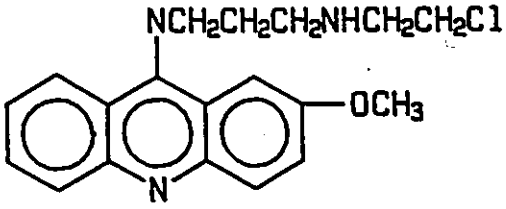
2-aminofluorene



2-acetylaminofluorene



7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE)



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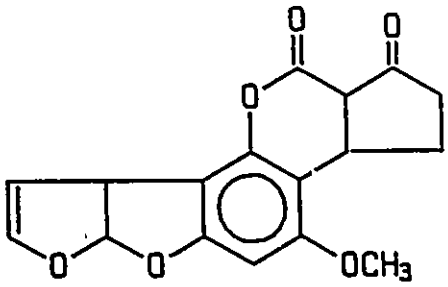
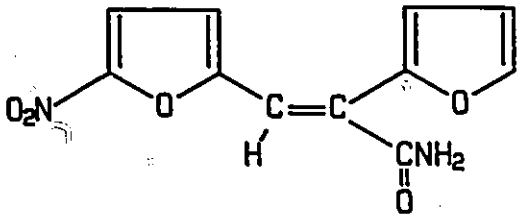


Aflatoxin B_1 (AFB_1)



2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide (AF2)

Figure 23. Mutagen Structures.
This figure shows the structures of several mutagens which are discussed in Chapter 3.

substitutions induced by AF at G:C sites were mainly (88%) G:C => T:A transversions and were umuC dependent (Bichara and Fuchs, 1985). AAF exhibited a similar mutagenic specificity in the lacI nonsense system (unpublished results cited in Miller, 1983). A significant proportion of G:C => A:T transitions and G:C => C:G transversions, in addition to G:C => T:A transversions, were recovered following AF treatment of M13 vectors containing the lacZ α-complementation gene segment (Gupta et al., 1988). However, in the study by Gupta et al. (1988), the similarity between the transitional changes which occurred in the AF spectrum and spontaneous base substitution mutations (characterized from unmodified vectors) led the authors to speculate that the G:C => A:T transitions might have arisen spontaneously. Although G:C => T:A transversions were also induced by AFB_1-2,3-dichloride (which reacts primarily at the N(7) position of guanine) in the lacZ α-complementing gene segment, almost 50 % of the base substitutions induced by this compound were G:C => A:T transitions and G:C => C:G transversions (Sambamurti et al., 1988). Similarly, BPDE which reacts at both the exocyclic N(2) position and the N(7) position of guanine induces G:C => T:A transversions (68 %) and G:C => A:T transitions (26 %) at G:C sites in the lacI gene of a pKM101 containing host (Gordon, Bernelot-Moens and Glickman, in preparation).

The observation that 1,8-NONP-induced G:C => T:A transversions are largely dependent on functions encoded by plasmid pKM101 suggests that adenine is incorporated opposite the adduct during error-prone DNA synthesis. Several different mechanisms might

explain the fact that adenine is preferentially incorporated opposite the lesion: 1) depurination could occur at the site of the lesion followed by incorporation of adenine opposite the apurinic site; 2) adenine might be preferentially inserted across from a stable but noninformational lesion without proceeding through an apurinic site intermediate; 3) the DNA adduct might be capable of directing the incorporation of adenine; and 4) the adenine might have been encoded by bases flanking the adduct by means of transient misalignment mechanisms. These possibilities are discussed in greater depth below.

a) Apurinic (AP) site intermediates. It is well established that AP sites are mutagenic (reviewed in Loeb and Preston, 1986). Studies which have examined the mutational specificity of depurination/depyrimidination have shown that the base most commonly incorporated opposite such sites is adenine, and that induction of the SOS response is required for mutagenesis (Schaaper and Loeb, 1981; Kunkel, 1984). Kunkel (1984) sequenced 79 base substitution mutations resulting from depurination in the α-complementation segment of the lacZ gene and showed that the specificity of incorporation of bases opposite aguaninic sites was adenine (56 %), thymine (29 %), and guanine (15 %) (Kunkel, 1984). Recently, on the basis of dNTP turnover opposite AP sites in vitro, Hevroni and Livneh (1988) have predicted the ratio for misincorporation of bases opposite aguaninic sites. These values (adenine (65 %), thymine (20%), guanine (15 %)) are remarkably close to those observed in vivo by Kunkel (1984).

Miller (1983), and Loeb (1985) have proposed that a common property of bulky adducts could be their ability to cause depurination at the site of a stalled replication complex. This might explain the tendency of such lesions to induce G:C => T:A transversions. For compounds which react with the N(7) position of guanine, such as AFB_1-2,3-dichloride (Swenson et al., 1975) and BPDE (Lobanenkov et al., 1986), this is an extremely plausible mechanism. Covalent binding to N(7) of guanine is believed to result in the formation of unstable quaternary amines; subsequent hydrolysis of the N-glycosyl bond yields AP sites (Loeb and Preston, 1986). On theoretical grounds, this explanation is less satisfactory for compounds which react at the C(8) position of guanine since adduction at C(8) of guanine does not alter the charge in the purine ring system, and therefore is less likely to enhance the lability of the N-glycosylic bond.

A comparison of the specificity of base substitutions induced by depurination with those caused by 1,8-NONP suggests that the changes induced by 1,8-NONP are not the result of depurination. Although adenine is the base which is most frequently incorporated opposite aguaninic sites, thymine and guanine are also inserted at relatively high frequency (Kunkel, 1984; Hevroni and Livneh, 1988). If AP sites were important intermediates for base substitution mutation induced by 1,8-NONP, it would be expected that about 30 to 40 % of the base substitutions occurring at G:C sites would be G:C => A:T transitions and G:C => C:G transversions. Among the base substitutions recovered from EE125 and CM6114, this is clearly not

the case: G:C => T:A transversions represent about 92 %, G:C => A:T transitions 5 %, and G:C => C:G transversions 3 % of the base substitutions formed at G:C sites.

In a separate study we have determined the mutational spectrum of the 5-nitrofuran derivative 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide (AF2) in the portion of the lacI gene encoding the DNA binding domain (see appendix 2 for details). These experiments were done in an E. coli strain which was deficient in excision repair, and contained the plasmid pKM101. On the basis of the observed specificity of mutation, we have postulated that this compound might act through an apurinic site intermediate, and have proposed a pathway for adduct formation at the N(7) position of guanine which is consistent with this hypothesis. Table 26 compares the distribution of base substitution mutations induced by AF2 and 1,8-NONP in the lacI gene of E. coli strains which contain the plasmid pKM101. While all spectra contain a high frequency of G:C => T:A transversions, the base substitutions induced by AF2 include a high proportion of G:C => A:T transitions as well. This comparison is particularly relevant since the same mutagenic system (lacI) has been used in both cases. Table 27 shows a comparison between the ratio of adenine, thymine, or guanine expected opposite an aguaninic site (Kunkel, 1984), and that actually observed in the 1,8-NONP and AF2-induced base substitution spectra. While the distribution of AF2-induced changes is not statistically different from those formed at putative aguaninic sites, the distribution of 1,8-NONP-induced base substitutions are quite distinct ($P<0.001$ in all cases).

Table 26. Comparison of base substitution mutations induced in the lacI gene by 1,8-NONP and AF2.

Treatment Strain	1,8-NONP CM6114 (pKM101, $_{\Delta}$uvrB)	1,8-NONP CM6114 (pKM101, $_{\Delta}$uvrB)	1,8-NONP EE125 (pKM101)	AF2 TC3960 (pKM101, $_{\Delta}$uvrB)
Gene	lacI (NC^{+a})	lacI (WG^{a})	lacI (WG^{a})	lacI (NC^{+})
Mutation Class				
G:C => T:A	29	66	31	76
G:C => A:T	1	4	3	49
G:C => C:G	2	2	1	10
A:T => T:A	4	7	1	9
A:T => C:G	1	1	2	0
A:T => G:C	0	0	1	1
Total	37	80	39	145

Values given are the number of base substitutions of each type recovered in the lacI gene following treatment with either 1,8-NONP (this chapter) or AF2 (Appendix 2). Note that the AF2 study examined mutations in the initial 180 base pairs of the lacI gene.
[a] WG, base substitutions in the entire lacI gene; NC^{+}, base substitutions monitored in the initial 180 base pairs of the lacI gene.

Table 27. Base incorporation opposite aguaninic sites, or opposite guanine residues modified with either 1,8-NONP or AF2.

Treatment Strain	1,8-NONP CM6114	1,8-NONP CM6114	1,8-NONP EE125	AF2 TC3960	Aguaninic sites[b]
Gene	*lacI*(NC^{+a})	*lacI*(WG[a])	*lacI*(WG[a])	*lacI*(NC^{+})	*lacZα*
Incorporation opposite guanine					
Adenine	91%	92%	89%	56%	56%
Thymine	3%	5%	9%	36%	29%
Guanine	6%	3%	3%	8%	15%

[a] WG refers to the entire *lacI* gene. NC^{+} refers to the initial 180 base pairs of the *lacI* gene.
[b] Data from Kunkel (1984).

b) Stable noninformational sites. A stable DNA adduct might be noninformational to the DNA polymerase acting at a replication fork. For chemicals which produce bulky adducts this would probably be most likely when adduction occurs at 1) a site which is directly involved in base-pairing; or 2) a major groove site followed by an anti => syn conformational change which would rotate the adduct moiety into the centre of the helix. In the case of 1,8-NONP, which reacts primarily at the C(8) position of guanine, the latter possibility should be considered. No studies to date have examined the conformation of dG-C(8)-ANP in DNA; however, the structure of DNA modified at the C(8) of guanine residues by AF and AAF is informative. Conformations of DNA modified with either AAF (Fuchs et al., 1976; Santella et al., 1981; Hingerty and Broyde, 1982; Broyde et al., 1990) or AF (Broyde and Hingerty, 1983; Norman et al., 1989b; Broyde et al., 1990), which contain a syn guanine adduct, have been proposed. While the syn conformation is the major energetically stable structure possible for AAF adducts, a variety of low energy conformations have been proposed for AF adducts which contain guanine in the more conventional anti form (Broyde and Hingerty, 1983). It must be stressed that these studies reflect the conformation of dG-C(8) adducts in double stranded DNA; the structure of adducts at the replication fork, where the adduct environment includes a doubled stranded-single stranded DNA junction in addition to the protein components of the replication complex, is unknown.

The fact that other dG-C(8) adducts can induce anti => syn conformational changes suggests that a similar phenomenon could be

feasible for dG-C(8)-ANP. In the context of the present discussion it might be imagined that such a rotation around the N-glycosylic bond would insert the pyrene moiety into the minor groove and create a site which is uninstructional to DNA polymerase. Presuming that the response to such a site is the incorporation of adenine (Miller, 1983; Rabkin and Strauss, 1984), this could explain the preponderance of G:C => T:A transversions observed in the mutational spectrum.

c) Miscoding Lesions. It is possible that no site is absolutely uninstructional to the DNA polymerase. If a dG-C(8) adduct is capable of pairing with noncomplementary bases, then it might also be capable of acting as a miscoding lesion during DNA synthesis. Several studies have described G:A base pairs in which the guanine residue is either unmodified, or modified at the C(8)-position with arylamine derivatives. These structures are shown in Figure 24 and described in more detail below.

In the normal anti guanine configuration, the hydrogen bonding groups of guanine (O^6, H-N(1), and H_2N^2) form Watson-Crick base-pairs with cytosine. Since adduction at C(8) of guanine does not interfere directly with the hydrogen bonding groups of guanine, it is possible that the adduct would also form stable base pairs with cytosine (Figure 24 (c)). This is supported by the observation that cytosine is preferentially incorporated opposite AAF (Rabkin and Strauss, 1984) and AF adducts (Michaels et al., 1987) during DNA synthesis in vitro. Loechler (1989) has proposed that the bulky constituent of C(8) adducts might be capable of shifting anti guanine towards the major groove, a perturbation termed adduct-induced base

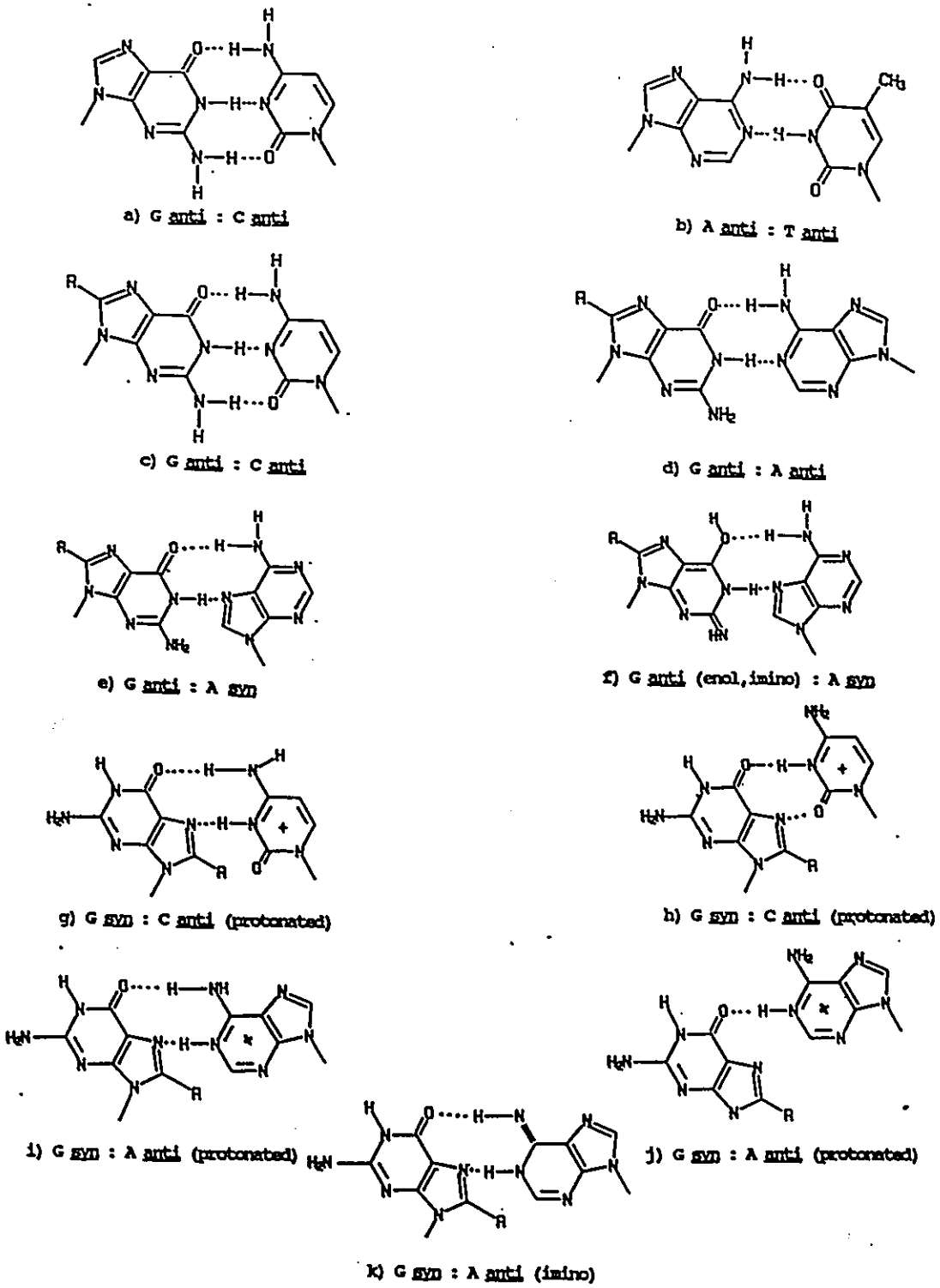


Figure 24. Potential G:A and G:C base pairs when G is modified at C(8).
R is a chemical which forms an adduct at C(8) of guanine.

wobble. This would result in the guanine becoming more "pyrimidine-like" and might facilitate the formation of a G:A mismatch (Figure 24 (d)). Evidence for the structure of this G:A mismatch in unmodified DNA has been reported by Kan et al. (1983); the O^6 and H-N(1) of the guanine residue is paired to the exocyclic amino group and N(1), respectively, of adenine. Recently, the crystal structure of a stable G(anti):A(syn) form has been characterized by Brown et al. (1986) (Figure 24 (e)). The guanine in these studies was not modified. With the O^6 and H-N(1) of the modified guanine paired to H_2N^2 and N(7) of adenine (syn), this structure contains the same hydrogen bonding pairs as would occur with the guanine in the rare (enol, imino) tautomer (Figure 24 (f)).

In unmodified DNA it is unlikely that G (syn) would occur in the template strand (Topal and Fresco, 1976). However the observation that other dG-C(8) adducts induce rotation about the N-glycosylic bond suggest that this might also occur in 1,8-NONP modified DNA. Recently, computer modelling (Broyde et al., 1990) and NMR (Norman et al., 1989b) studies have suggested that C(8) adducts formed with AF can pair with either C or A when the modified guanine residue is in the syn conformation. All of the possible structures require that the base which is paired to the adduct be in rare ionized or tautomeric forms. However, Quigley et al. (1986) and Sowers et al. (1987) have suggested that protonation of bases can occur more frequently in DNA duplexes than was anticipated from the pK of free bases in solution. Strictly on the basis of hydrogen bonding potential, the structure shown in Figure 24 (i) is predicted

as most likely to form a G(syn):A (protonated) mismatch. However, NMR studies with an AF-modified 11-mer containing a G(adduct):A mismatch have shown that the O^6 of the modified syn guanine in fact forms a hydrogen bond with the H-N(1) of protonated adenine (Figure 24 (j)) (Norman et al., 1989b). Similar structures can be proposed for G(syn):C(protonated) pairs (Figures 24 (g) and 24 (h)). Critical to the stability of all the base pairs involving a syn adduct is the ability of the hydrophobic aminofluorene moiety to wedge into the minor groove so as to minimize its exposure to the solvent (Broyde et al., 1990). The amino-8-nitropyrene moiety of the major adduct formed with 1,8-NONP contains 4 fused 6-membered aromatic rings as well as a nitro group; whether the minor groove could accommodate such a large group is unknown. A final G (syn) structure which would give rise to a G:A mispair is shown in Figure 24 (k). This structure contains A (anti) as the imino tautomer with O^6 and N(7) of the adducted syn guanine pairing with H-N^6 (imino) and H-N(1) of adenine (Topal and Fresco, 1976).

d) Transient Misalignment. In vitro experiments have provided evidence that base substitution mutations can be templated by adjacent bases due to transient misalignment of the template and primer strands during DNA synthesis (Kunkel and Soni, 1988). Two possible mechanisms of misaligned templating are illustrated in Figure 25. 1) The template strand could slip back upon the primer such that the base 5' to the adduct acts as the template for the incorporation of the next base. If this were followed by realignment of the two strands and resumption of DNA synthesis at the base 5' to

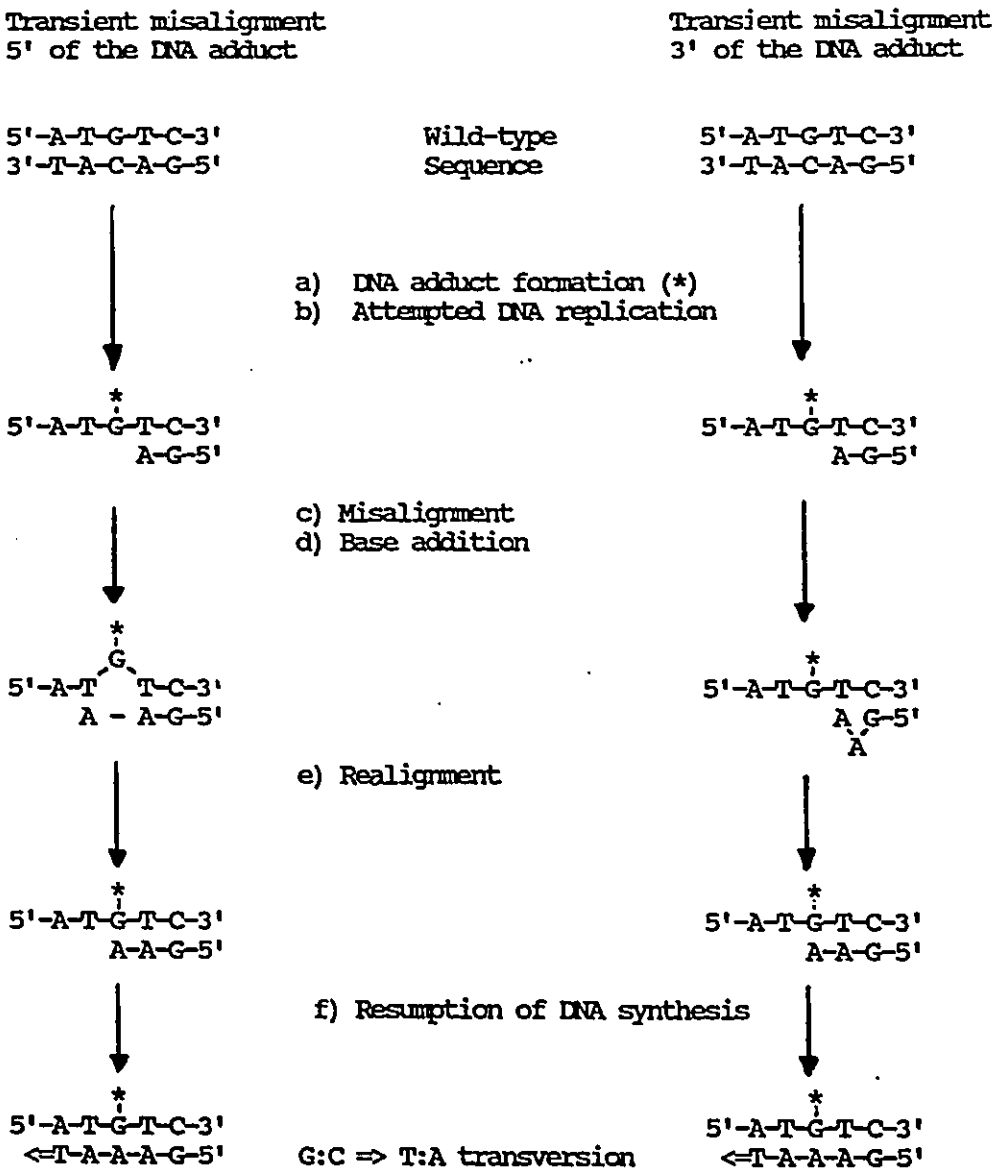


Figure 25. Mechanisms of transient misalignment.
The example given here is for a G:C => T:A transversion. These pathways are described in the text.
* = chemical which forms DNA adduct.

the adduct, the daughter sequence would be derived by a mechanism independent of the coding capacity of the adduct (Figure 25a) (Kunkel and Alexander, 1986). 2) Templating by the base 3' to the adduct might occur if the primer strand slipped back upon the template subsequent to incorporation opposite the 3' base. This would result in incorporation of a second base which, following realignment, would be situated opposite the adduct (Figure 25b). Although the presence of an adduct is not necessary for misalignment mechanisms, the ability of a bulky lesion to slow down replication, and subsequently increase the period in which the misalignment/realignment steps could occur, might be expected to facilitate the process.

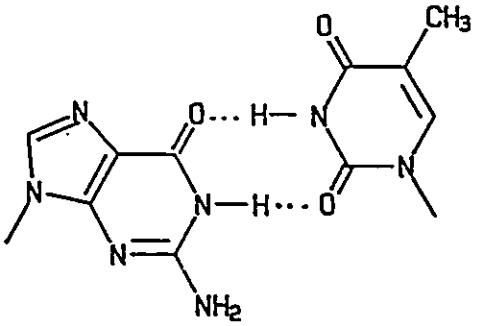
The site specificity of the G:C => T:A transversions recovered following 1,8-NONP treatment is not consistent with misalignment mechanisms. There is a notable lack of nearest base influence in the 1,8-NONP-induced transversions (Table 25). The transient misalignment mechanisms would predict that a large proportion of the base substitutions occur at the 25 detectable G:C => T:A transversion sites (Table 24) which are flanked by thymine. However, only one third of the 1,8-NONP-induced G:C => T:A transversions characterized in this study occurred at these sites.

3.4.2.2 G:C => A:T Transitions

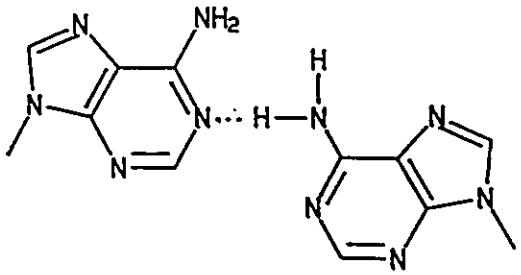
Following 1,8-NONP treatment, the majority of base substitution mutations (12/23) recovered from strains NR6112 or NR6113, which do not contain the plasmid pKM101, were G:C => A:T transitions. In contrast, only 7 G:C => A:T changes were among the 119 base substitution mutations recovered from the pKM101-containing

strains EE125 and CM6114. Since G:C => A:T transitions are the most common base substitution events found in collections of spontaneous mutants (Table 19; Halliday and Glickman, unpublished results), it is possible that lacI⁻ mutations of this type obtained from 1,8-NONP-treated cultures are also of spontaneous origin. This is particularly likely in the NR6112 collection where the frequency of base substitution mutations was not increased above the spontaneous level (Table 20). Although base substitutions appeared to be induced by 1,8-NONP in the uvrB⁻ strain NR6113, they comprise a relatively small proportion (8 %) of the induced spectrum in this strain. Fully 63 % (12/19) of the G:C => A:T transitions recovered in all of the E. coli strains following 1,8-NONP treatment occurred at sites which have previously been identified in spontaneous collections (Coulondre et al., 1978; Halliday and Glickman, unpublished results; Gordon and Glickman, unpublished results). There are three positions in the lacI gene (104, 419, and 959) where deamination of 5-methylcytosine (produced at 5'-CCAGG-3' sequences by dcm methylase) might yield thymine and thus lead to G:C => A:T transitions. Four mutants contained G:C => A:T transitions which occurred at two of these sites (3 at position 104; 1 at position 959). Only 2 out of 19 G:C => A:T transitions occurred at sites where transient misalignment mechanisms might produce the desired mutation. One of these (at position 90) has previously been postulated to be a site where misalignment might occur (Schaaper, 1988).

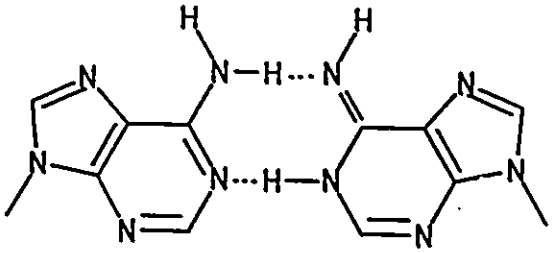
In unmodified DNA, thymine is misincorporated opposite G at relatively high frequency (Fersht et al., 1982) to form a G:T base-



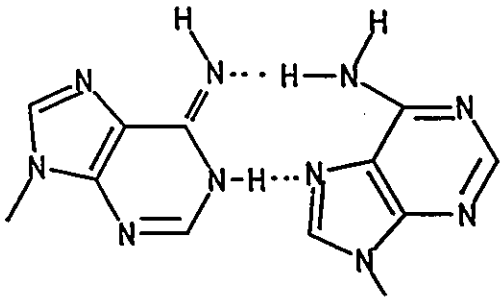
a) G anti : T anti



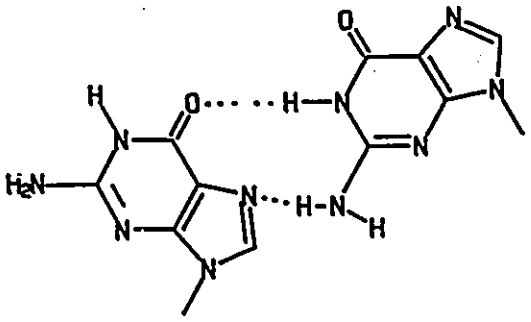
b) A anti : A anti



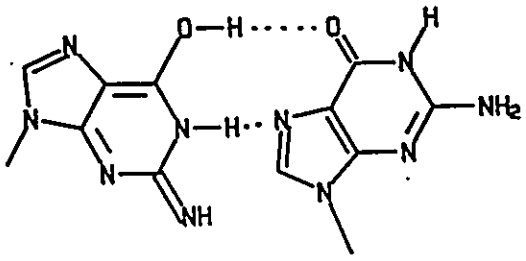
c) A anti : A anti (imino)



d) A anti (imino) : A syn



e) G syn : G anti



f) G anti (enol,imino) : G syn

Figure 26. Potential A:A, G:T, and G:G base pairs.

pair (Figure 26 (a)) (Brown et al., 1985; Kneale et al., 1985). This process might be influenced by modification of guanine at the C(8) position. It has been shown that relative to the G:A mismatch, G:T mismatches are: 1) excised much more efficiently by the 3' => 5' exonuclease of DNA polymerase III of E. coli (Fersht and Knill-Jones, 1983); and 2) subject to more stringent recognition and repair by certain post-replicative mismatch repair enzymes (Dohet et al., 1985). Whether preferential correction of a G(adduct):T mismatch would occur more frequently than correction of a G(adduct):A mismatch is not known, but could account for the relatively small number of G:C => A:T transitions characterized in these studies.

3.4.2.3 A:T => T:A Transversions

A total of 8 A:T => T:A transversions were recovered from E. coli strains following 1,8-NONP treatment. All of these were formed in strains containing the plasmid pKM101. Massaro et al. (1983) have previously shown that 1,8-DNP is mutagenic in S. typhimurium TA102, which can revert as the result of base substitutions at A:T base pairs (Hartman et al., 1986). Mutations of this nature are consistent with the observation that dA or dT adducts are formed by 1,8-NONP in E. coli (Chapter 2). It is therefore possible that the A:T => T:A transversions are targeted. Since SOS processing opposite bulky lesions often leads to the preferential incorporation of adenine, the pKM101 dependence of these mutations suggests that the adduct occurs on adenine, since incorporation of A opposite thymine adducts would not yield mutations.

Possible mechanisms for the formation of A:T => T:A

transversions are similar to those already discussed in detail regarding G:C => T:A transversions. Potential A:A base-pairs are shown in Figure 26 (b-d): two of the potential pairs (c and d) contain A in the unfavored imino tautomeric form, while the third possibility (26 (b)) contains only a single hydrogen bond. None of the eight A:T => T:A transversions occur at sites which would produce this mutation by a transient misalignment mechanism.

3.4.2.4 Other base substitution mutations

A small number of other base substitution mutations were recovered from 1,8-NONP treated cultures. These include 5 G:C => C:G and 3 A:T => C:G transversions, and 1 A:T => G:C transition. Since these mutations are relatively rare in mutants obtained from 1,8-NONP treated cultures, and 6/9 occur in *uvrB*$^+$ strains, it is likely that several are of spontaneous origin. This is particularly true of the A:T => G:C mutation which could occur as a result of adenine deamination to yield hypoxanthine which base pairs with cytosine. Although the number of mutations of these types is too small to provide reliable information regarding site specificity, it is interesting to note that 8 out of these 9 can be accounted for by transient misalignment mechanisms. This raises the possibility that the nature of these changes was determined by the identity of one of the flanking bases.

The potential for the formation of G:A (or A:G) base-pairs which might give rise to A:T => C:G transversions has been discussed in depth above and the possible structures are shown in Figure 24. Structures of unusual G:G base pairs have been described (Drake and

Baltz, 1976; Topal and Fresco, 1976) and are shown in Figure 26 (e and f). Of these structures, the one shown in panel e, is more probable since it does not require a rare tautomeric form and is theoretically applicable to either a G(syn) or G(anti) template.

3.4.3 Frameshift Mutations

Frameshift mutations were induced by 1,8-NONP in all of the E. coli strains used in these experiments. The prominence of -1, -2 or +1 frameshift mutations in 1,8-NONP-treated cultures stands in sharp contrast to the very low spontaneous frequency of this type of mutation (Table 19; Schaaper et al., 1986; Halliday and Glickman, unpublished results). It is estimated (Table 20) that frameshift mutations were increased 6.7-fold in NR6112 (wild-type), 15.2-fold in strain EE125 (pKM101), 500-fold in NR6113 (uvrB$^-$), and 560 to 850-fold in CM6114 (pKM101, uvrB$^-$). The specificity of the frameshift mutations provides further evidence that the majority are the result of the mutagenic treatment. Ninety-six percent of the frameshift mutations collected following 1,8-NONP treatment involved the gain or loss of a G:C base pair. In contrast, spontaneous -1 frameshift mutations are just as likely to involve the gain or loss of A:T base pairs as G:C base pairs (Schaaper et al., 1986; Halliday and Glickman, unpublished results; Gordon and Glickman, unpublished results). In addition, about 98 % of the frameshift mutations characterized here involved the loss of nucleotides, while in spontaneous cultures the figure is 70-80 % (Streisinger and Owen, 1985; Halliday, Gordon and Glickman, unpublished results).

The ability of 1,8-NONP to induce frameshift mutations is

similar to the mutagenic activity of 1,8-DNP in S. typhimurium strains (Ames test). 1,8-DNP is extremely mutagenic in the S. typhimurium frameshift tester strains TA1537, TA1538, and TA98. In the forward mutation assays conducted in the present study, the majority of the frameshifts recovered (-1 at reiterated G:C sequences) were similar to those which can revert the hisC3076 locus of TA1537. This reversion hotspot contains a sequence of 5 contiguous guanine residues which revert by the loss of a single G:C base pair (Cebula and Koch, 1989). 1,8-DNP is mutagenic in TA96 (Massaro et al., 1983), which frequently reverts as the result of a -1 frameshift in a sequence containing 5 consecutive A residues (Hartman et al., 1986). Following 1,8-NONP treatment, a small number (13) of lacI$^-$ mutations arose as the result of the gain or loss of A:T base pairs. This is also consistent with the Ames test results.

There are some qualitative differences between the frameshift events recovered here and those scored in Ames tests. The tester strains TA98 and TA1538 contain the sequence 5'-CCGCGCGCGG-3' at the hisD3052 locus which frequently reverts to the wild-type by loss of GC:CG (Hartman et al., 1986). 1,8-DNP is even more active in strains containing this site than in TA1537. In contrast, only 8 (out of 295) frameshift mutations recovered in the lacI gene following 1,8-NONP treatment resulted from -(GC:CG) events. A possible explanation for this is that the structural requirements for sequences which produce -(GC:CG) mutations might be extremely stringent. Two types of sequences have been identified which yield -(GC:CG) frameshifts at high frequency, particularly following treatment with some chemicals:

1) sequences like the NarI restriction site (5'-GGCGCC-3') (Fuchs et al., 1981; Fuchs, 1983; Koffel-Schwartz et al., 1984; Fuchs et al., 1988); and 2) extended alternating GC sequences (Broyde and Hingerty, 1987; Fuchs et al., 1988; Freund et al., 1989) such as occur at the hisD3052 locus of TA98 (Hartman et al., 1986). There are no NarI sites in the lacI gene, nor is there an alternating GC sequence as long as the 8 nucleotides at the hisD3052 locus. The longest alternating GC sequence in the lacI gene is 6 nucleotides at position 790-795; two -(GC:CG) mutations were recovered at this site. It is possible that structural intermediates for -2 frameshifts are more stable when the alternating GC sequence is 8 nucleotides in length as opposed to 6 nucleotides in length. This difference in the nature of the target might account for the discrepancy between results obtained in the different experimental systems.

The high proportion of frameshift mutations which involve the gain or loss of a G:C base pair (96 %) is consistent with the possibility that the frameshifts were targeted by dG-C(8)-ANP. The spectrum of frameshift mutations obtained following treatment with 1,8-NONP is similar in some respects to those resulting from AAF adduction at guanine C(8). Fuchs and coworkers have characterized the mutational spectrum of AAF in the tetracycline resistance gene of plasmid pBR322 in several E. coli strains. The prominent class of mutations formed were frameshifts, which could be divided into two different types: 1) frameshifts in sequences of contiguous guanine residues, involving the loss of a single G:C base pair and occurring in a umuC dependent fashion; and 2) -(GC:CG) frameshifts at the NarI

restriction site which were umuC independent (Koffel-Schwartz et al., 1984; Fuchs et al., 1988).

3.4.3.1 -1 Frameshifts at G:C sites

Examination of the sites at which -1 (G:C) frameshift mutations occur illustrates the importance of reiterated DNA sequences. The likelihood of a frameshift mutation increases markedly with the number of contiguous guanine residues. Mutations were recovered at only 14 out of the 312 detectable frameshift sites in the lacI gene which contain a lone G:C base pair, and 26 out of the 81 detectable sites containing two consecutive guanine residues. However, frameshifts were recovered at all 23 NGGGN sites which can be detected, with an average of 7.2 events per site. One detectable NGGGGN site is present in the lacI gene; 29 frameshift mutations were recovered at this site following 1,8-NONP treatment. Frameshifts involving the loss of a G:C pair occurred preferentially at NGN and NGGN sites which contain a 5'-flanking T and/or a 3'-flanking A, and at NGGGN sites containing a 3'-flanking A. The dependence of 1,8-NONP-induced frameshift mutagenesis on reiterated sequences is similar to many spontaneous frameshift mutations (Streisinger and Owen, 1985) as well as those induced by AAF (Koffel-Schwartz et al., 1984), aflatoxin B_1 (Refolo et al., 1987; Sambamurti et al., 1988), BPDE (Bernelot-Moens, Glickman and Gordon, submitted), intercalating agents such as proflavin (Streisinger and Owen, 1985), and intercalating acridine compounds with alkylating sidechains such as ICR-191 (Calos and Miller, 1981). A model which might explain the propensity for frameshift mutations to occur in sequences with

repeated bases was first proposed by Streisinger and coworkers (Streisinger et al., 1966; Streisinger and Owen, 1985). These workers postulated that reiterated sequences stabilize replication intermediates derived from slippage of one strand relative to the other, causing one or more extrahelical bases to bulge from the DNA duplex. If an extrahelical base occurs on the template strand during DNA synthesis, then loss of a base is predicted; conversely, an extrahelical base on the primer strand would lead to the addition of a base. The fact that virtually all of the frameshift mutations induced by 1,8-NONP at G:C sites (almost 98 %) result from the loss of bases suggests that an extrahelical base is produced on the template strand.

The presence of bulky lesions during DNA synthesis pose a fundamental problem for slippage models: in order for slippage to occur, a portion of the reiterated sequence must be copied accurately onto the nascent strand prior to slippage. However, the presence of a bulky adduct impedes replication and clearly makes it difficult for the nascent strand to be copied at all. The observed specificity of the frameshift mutations induced by 1,8-NONP suggest an "incorporation-slippage" model (Figure 27) which circumvents this problem. Central to this model is the potential of C(8) guanine adducts to form base pairs with cytosine. Although the appropriate experiments have not been done with 1,8-NONP or 1,8-DNP, the effect of AF and AAF adducts on DNA synthesis in vitro has been measured in several laboratories. AAF adducts terminate progression of E. coli DNA polymerase I (Moore et al., 1980; Rabkin and Strauss, 1984), phage T7

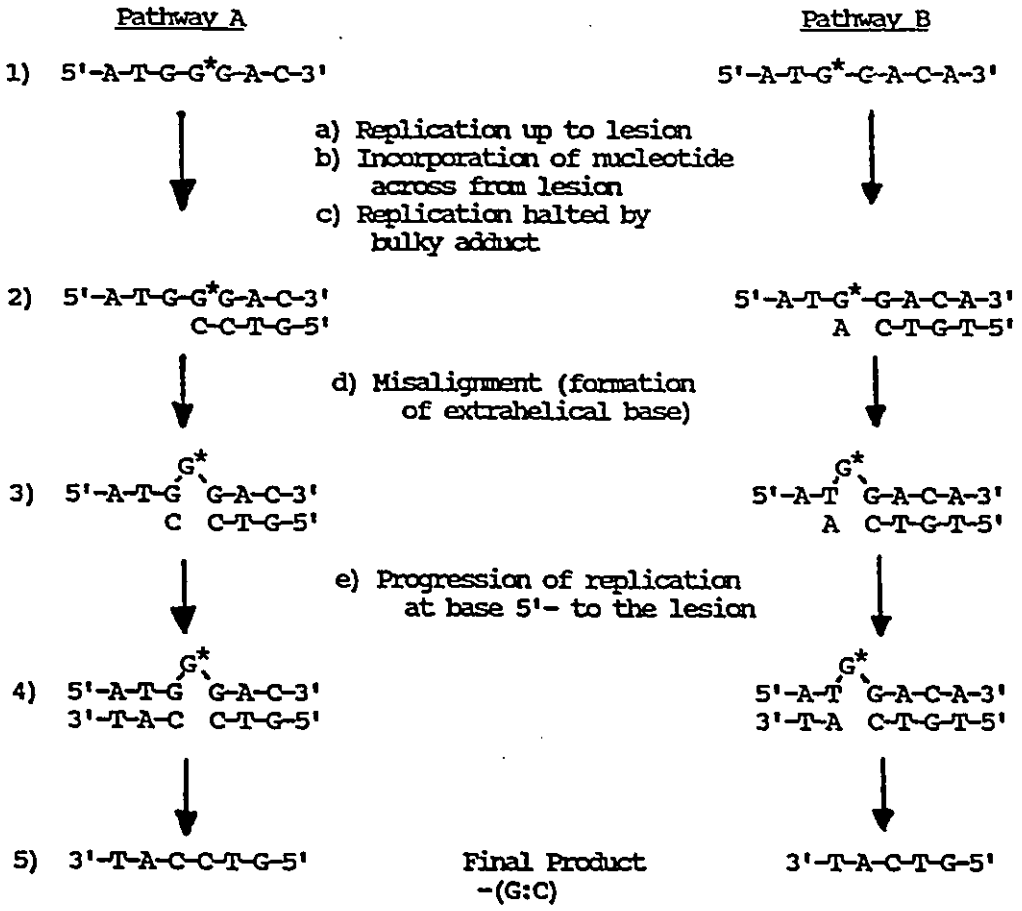


Figure 27. Incorporation-slippage model for 1,8-NONP induced frameshift mutagenesis.
1) Adduct formation occurs at the C(8) position of guanine. DNA polymerase attempts to incorporate bases across from the lesion during DNA synthesis. The adducted base is predicted to be capable of coding properly for cytosine (pathway A) or miscoding for adenine (pathway B). Following incorporation of the base, the bulky adduct interacts with the replicational complex to halt its progression. 2) Stalling at the site of the lesion allows time for slippage to occur (possibly aided by a conformational change facilitating the formation of an extrahelical base). 3) If the base 5' to the lesion pairs properly with the newly incorporated base then the stability of this structure will be enhanced. Pathway A predicts that this would occur only in runs of G:C bases. Misincorporation of adenine (pathway B) could lead to a -1 frameshift at guanines with a 5' flanking thymine. 4) Progression of the replicational complex would resume at the base 5' to the lesion leading to the final product (5) which is a -1 frameshift.

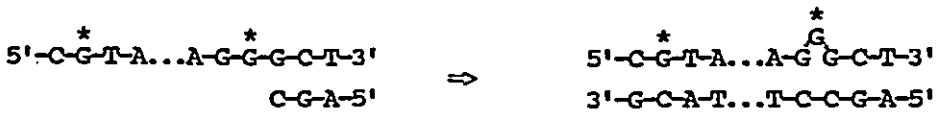
DNA polymerase (sequenase) (Sahm et al., 1987), AMV reverse transcriptase (Moore et al., 1982) or mammalian polymerase α (Moore et al., 1981) either 1 base before the site of the lesion or following incorporation of a nucleotide opposite the lesion. The bases incorporated opposite the AAF lesion were C ≥ A >> G or T (Rabkin and Strauss, 1984). The majority of the synthesis past the site of the AF lesion occurs with the accurate incorporation of C (Michaels et al., 1987), consistent with the hypothesis that AF adducts can form G:C base pairs. When a wrong base is inserted, that base is usually adenine (Sahm et al., 1987). Rabkin and Strauss (1984) have proposed that incorporation of A opposite the AAF adduct occurs when the adduct rotates into a noninformational syn conformation, while the incorporation of C occurs when the adducted base assumes the informational anti conformation (Rabkin and Strauss, 1984). With the C(8) adduct of 1,8-NONP, either of these outcomes might be expected based on: 1) the theoretical ability of anti dG-C(8) adducts to form normal Watson-Crick base pairs with cytosine (Figure 24 (c)); and 2) the evidence that 1,8-NONP-induced base substitution mutations are primarily the result of adenine insertion opposite the adduct. If, following incorporation of a base opposite the lesion, the progression of the replication fork were halted by the presence of the bulky adduct (perhaps due to dissociation of the polymerase from the replication fork), more time would be available for strand slippage. Such slippage of the template strand would force the adducted base into an extrahelical configuration and would be stabilized if the base which had been incorporated opposite the

lesion paired in a stable fashion with the base 5'- to the adduct. Progression of DNA synthesis could then occur from the base pair vicinal to the bulky lesion.

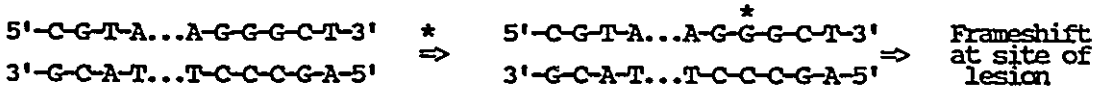
This model predicts that incorporation of C opposite the lesion might lead to frameshifts if the adduct were present in the following sequence contexts: NGG*N, NGG*GN, NGGG*N, NGG*GGN, NGGG*GN, and NGGGG*N (G* is an adduct). If adenine were incorporated then the relevant sequences would be TG*N, TG*GN, TG*GGN, and TG*GGGN. With the exception of TG*GGN all of these sequences are consistent with the experimental results obtained here. Note that incorporation of C without slippage would not cause a mutation while incorporation of A without slippage would yield a G:C => T:A transversion.

The efficiency with which frameshift mutations form will be determined in part by the ability of the sequence to accommodate an extrahelical base. Favorable intrastrand stacking of flanking purine bases (Saenger, 1984) might favour sequences such as RG*R (where R = purine). In this context it is notable that sequences such as NGG*AN and NGGG*AN are favoured frameshift sites. This fact, combined with the observation that frameshifts occur in runs of G might also be interpreted as supporting a mechanism (Figure 28a) which entirely emphasizes the ability of adducts in purine runs to become extrahelical due to favorable intrastrand stacking between the flanking purines (and possibly the aromatic pyrene nucleus if the adducted guanine was rotated into the syn configuration). While this mechanism can accommodate most of the sequences at which frameshift

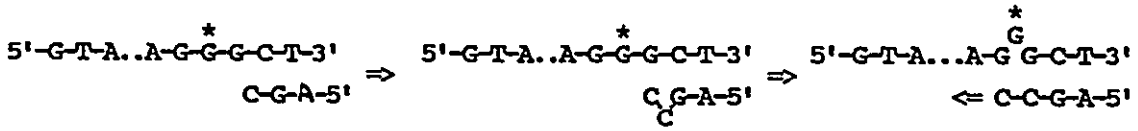
a) Intrastrand stacking between flanking purine residues



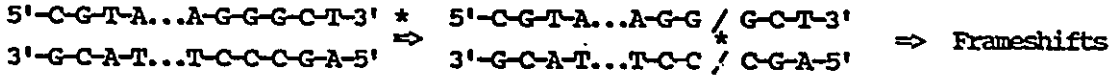
b) Preferential reactivity of guanine residues within reiterated sequences



c) Replicational stuttering at base 3'- to the adduct



d) Intercalation of pyrene moiety



* = chemical which forms adduct

Figure 28. Alternate mechanisms of frameshift mutation

mutations were characterized following 1,8-NONP treatment, it would also predict that mutations occur at NAG*AN , NAG*GN, and NAG*GGN sites. The fact that frameshift mutations were not recovered at these latter sites argues against the possibility that the specificity of 1,8-NONP-induced frameshift mutations can be explained solely by the ability of certain sequences to accommodate extrahelical bases.

A number of other models might account for the ability of 1,8-NONP to induce frameshift mutations. These are shown in Figure 28 and discussed briefly below. The observed specificity of frameshift mutations might be due to the preferential reaction of 1,8-NONP with bases within monotonous runs of guanine (Figure 28b). Studies with other compounds however suggest that this might be possible. Adduct formation with AFB_1 at the N(7) position of guanine occurs up to 10 times more frequently in runs of guanine than at isolated guanine residues (Muench et al., 1983; Misra et al., 1983; Sambamurti et al., 1988). Many, but not all of the hotspots for DNA adduct formation were also found to be mutation hotspots (Sambamurti et al., 1988). In contrast, AAF binding to DNA was found to be nonrandom but did not occur preferentially in runs of guanine. No correlation between the damage distribution spectrum, and the mutational spectrum has been observed for this mutagen (Fuchs, 1983; Koffel-Schwartz et al., 1984). With BPDE, adduct formation at the exocyclic N^2 position occurs within runs of G (Boles and Hogan, 1986), while binding to the N(7) position occurs preferentially at guanines preceded by a pyrimidine (Lobanenkov et al., 1986). At the

present time no damage distribution data are available for 1,8-NONP.

Figure 28c shows a base reiteration model similar to that shown in Figure 25b. In this mechanism the incorporation of cytosine into the nascent strand occurs as a result of replicational stuttering due to interaction of the adduct with the replicational complex in front of the lesion (Sakore et al., 1979). The cytidine on the 3' free end of the primer strand produced by such stuttering could hydrogen bond to the guanine immediately 5' to the adducted base on the template strand, creating the frameshift. This model is attractive since it does not assume that the adducted base has templating properties but since there is a requirement for 3 or more contiguous guanines, it fails to account for the significant number of frameshift mutations which occur at NGGN sites.

The fact that 1,8-DNP and 1,8-NONP are large, planar, fused aromatic ring structures suggests that they might be capable of acting as intercalating agents (Figure 28d). However, a great deal of experimental evidence argues against this possibility. 1) Intercalating agents induce both losses and additions of bases (Calos and Miller, 1981; Streisinger and Owen, 1985), while the vast majority of frameshift mutations induced by 1,8-NONP are due to the loss of bases. 2) In S. typhimurium, intercalating agents are more mutagenic at the frameshift locus hisC3076 (strain TA1537) than at the hisD3052 locus (TA1538 or TA98) (Rosenkranz and Mermelstein, 1983). However, 1,8-DNP is an extremely potent mutagen at both loci and in TA98 is more than 3 orders of magnitude more mutagenic than the potent intercalating compound ICR-191 (comparative data from

Rosenkranz and Mermelstein, 1983; Sambamurti et al., 1988). 3) In Ames tests, nitropyrenes are only weakly mutagenic in tester strains which are proficient in excision repair capability (Table 1). In the present studies the frequency of 1,8-NONP-induced frameshift mutations is also reduced considerably in uvrB$^+$ strains. In contrast, several intercalating agents induce frameshift mutations as effectively in uvrB$^+$ as in uvrB$^-$ strains (Rosenkranz and Mermelstein, 1983). 4) the requirement that 1,8-DNP be acted upon by acetyltransferase in order to be maximally mutagenic (Table 4) provides evidence that mutagenicity is mediated through reactive hydroxylamino or acetoxyamino species which give rise to DNA adducts. Thus it appears that covalent binding to DNA is required for 1,8-DNP and 1,8-NONP-induced mutagenesis, and that the specificity of mutation is clearly different from that which has been observed for mutagens whose mutagenic activity is the result of intercalation.

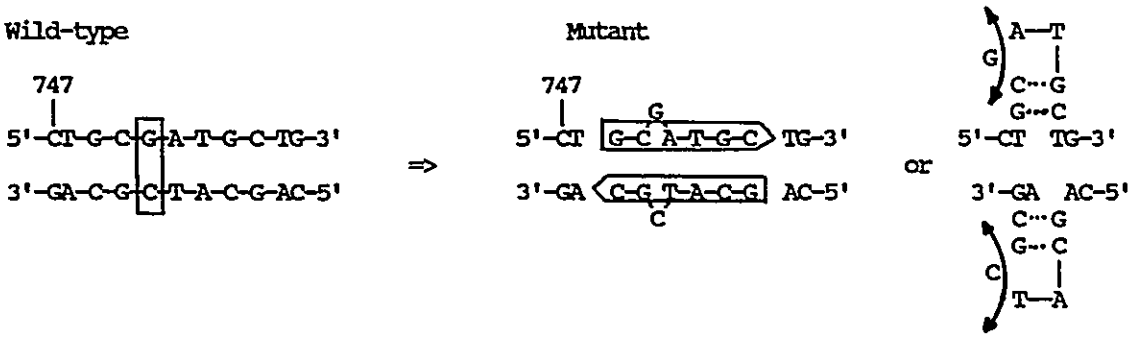
All but four frameshift mutations recovered following 1,8-NONP treatment can be accounted for satisfactorily by the incorporation-slippage model shown in Figure 27. Mutants L22, M22, M33, M72 are all due to the loss of a lone G:C base-pair and cannot be explained easily by simple slippage mechanisms. A number of studies have shown that both direct and inverted repeat sequences might provide an opportunity for the formation of misaligned intermediates which can be processed to produce base substitution, deletion and frameshift mutations (Ripley, 1982; Ripley and Glickman, 1983; Glickman and Ripley, 1984). In order to determine whether such sequences might contribute to these 4 frameshift mutations, the DNA

sequence surrounding the mutational sites was examined more closely, and the free energy of potential DNA hairpins was determined as described in section 3.3.2.10. Speculative explanations for these mutations are given in Figures 29 and 30; details are provided in the captions to these figures.

The free energy of possible secondary structures was evaluated using the Zuker algorithm (Zuker and Steigler, 1981). For the present application this has some limitations: 1) the sequence which is folded is not the DNA sequence, but rather the corresponding RNA sequence; 2) the degree to which a bulky adduct influences the free energy of given structures is unknown; and 3) since the program is a RNA single strand folding program, competing DNA duplex structures are not accounted for. Nevertheless, this program provides valuable information regarding the feasibility of certain structures.

The enzymatic requirements for bulky adduct frameshift mutagenesis are unclear. Work by Fuchs and coworkers suggests that AAF mutagenesis at reiterated sequences is dependent on functions encoded by umuC (Koffel-Schwartz et al., 1984), and is enhanced by functional mismatch repair capability (Granger-Schnaar et al., 1986). Frameshift mutation induced by AFB_1 is enhanced by mucAB encoded functions, particularly following SOS induction (Refolo et al., 1987); however, some mutagenesis occurred even in mucAB$^-$ strains, a phenomenon that these workers attributed to functions encoded by the endogenous umuCD locus. Studies with both AAF and AFB_1 have shown that the damage can be efficiently repaired by the uvrABC

a) Mutant M72 at position 751



b) Mutant L22 at position 927

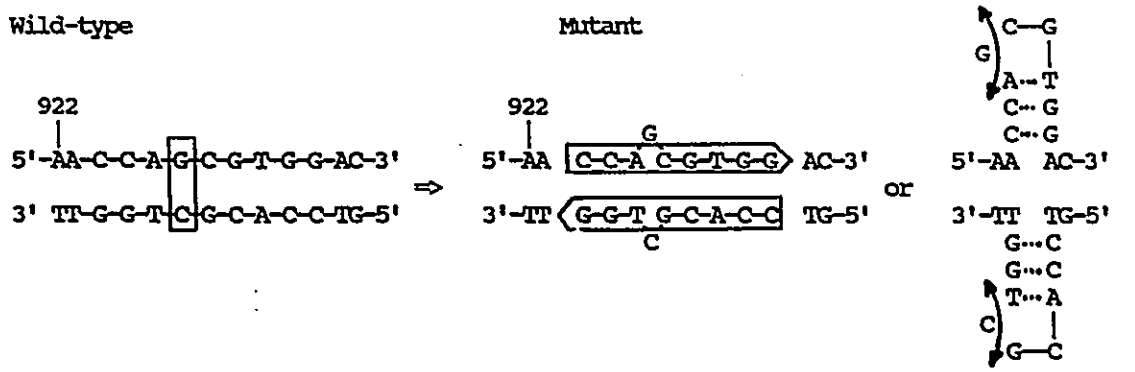
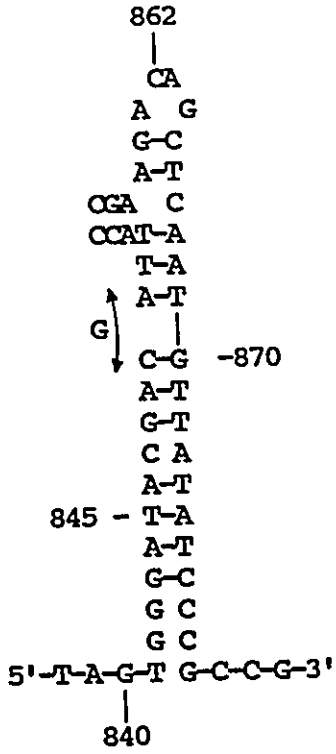


Figure 29. -(G:C) frameshift mutations: Mutants M72 and L22. Mutant M72 and L22 arose by loss of a G:C base pair at positions 751 and 927, respectively. Upon close examination of the surrounding sequence it is obvious that these mutations occur at similar sites. In each case the mutated sequence shows remarkable symmetry: 1) in M72, the sequence 5'-GCATGC-3' occurs on both the noncoding and coding strand, while in L22 the sequence is 5'-CCACGTGG; 2) in both cases there is a central axis of symmetry with the sequence on each side being precisely complementary; 3) finally, the G:C base-pair which is deleted to yield the resultant structure is one base removed from the axis of symmetry in each mutant (at position 751 in M72 and position 927 in L22). The mechanistic basis for these rare frameshift mutations is unknown. However, the remarkable similarity between the sites at which these two mutations occurred strongly suggests a role for these unique DNA sequences in frameshift formation.

a) Mutant M22 at position 851
Mutant M22 results from the loss of a G:C base pair at position 851 of the lacI gene. This mutant might have been templated by a nearby inverted repeat.



b) Mutant M33 at position 664
Mutant M33 results from the loss of a G:C base-pair from position 664 of the lacI gene. No palindromic structures can account for this mutation. However, it might have been templated by a 5 base direct repeat at position 692 or a 4 base repeat at position 633. In each of these cases the presence of a bulky DNA adduct might have increased the opportunity for strand slippage.

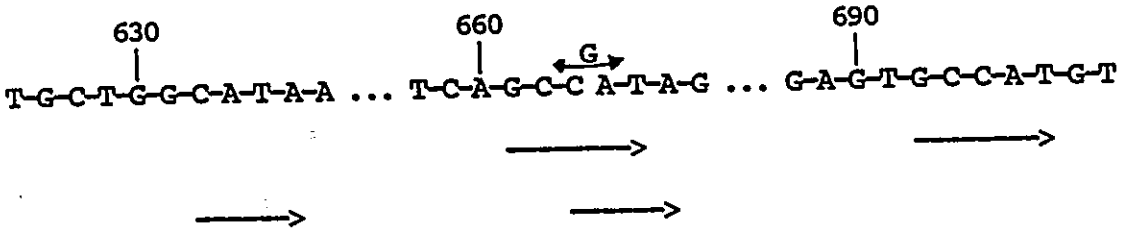


Figure 30. -(G:C) frameshifts: Mutants M22 and M33.

exinuclease. While the presence of excision repair is important in reducing the total amount of damage, it does not appear to influence the distribution of frameshift mutations (Koffel-Schwartz et al., 1984; Fuchs and Seeburg, 1984; Refolo et al., 1987).

In the present study it is difficult to determine the extent to which frameshift mutagenesis is dependent on error-prone repair functions. Relative to the otherwise isogenic strains lacking plasmid pKM101, the frequency of frameshift mutations in CM6114 and EE125 were increased only moderately (2 to 4-fold (Table 20). Thus the influence of pKM101 on frameshift mutations appears to be somewhat weaker than on base substitution mutations. In the context of the incorporation-slippage model presented here, this might be explained by considering the base from which replication resumes: to produce base substitution mutations, SOS functions facilitate progression of replication from a base-pair (adduct:base) at the site of the bulky lesion; in contrast, the frameshift model (Figure 27) predicts that following slippage and bypass of the lesion, DNA synthesis resumes from a Watson-Crick pair adjacent to the adduct. The combined effect of DNA synthesis resuming from a normal base-pair, and being somewhat removed from the steric hinderance of the bulky lesion might diminish the dependence of frameshift mutagenesis on SOS encoded functions. However, in order to draw more definitive conclusions, it would be necessary to determine the mutational specificity in a umu$^-$ strain.

The frequency of frameshift mutations is elevated by almost two orders of magnitude in strains which are deficient in excision

repair. It is likely that the principal effect of reduced excision repair is to elevate both the total adduct burden and the mean adduct residence time, thereby increasing the probability that the appropriate replication or repair enzymes might encounter a lesion in a sequence capable of giving rise to a frameshift event.

3.4.3.2 Other Frameshift Mutations

A small number of other frameshift mutations were recovered apart from those already discussed. These include -1 frameshifts at A:T base pairs, + 1 frameshifts at both A:T and G:C sites, and -2 frameshifts at alternating G:C sequences. The possible origin of these mutations is discussed below.

a) +(G:C) mutations. The addition of a G:C base pair occurred at three sites, positions 90-92 (5'-ACGCGGGAAAC-3'), 132-134 (5'-ACGCGGGAAAA-3'), and 604 (5'-CCGAGACAG-3'). It is notable that the sites at position 90-92 and 132-134 share 10 bases of homology at the site where the +1 mutation occurred, and that in each case the guanine run to which a base was added (underlined) is flanked on the 3' side by a run of adenine residues. The similarity is striking and might relate to the increased propensity of such sequences to support misalignment/realignment slippage events. The +(G:C) mutation at position 604 occurred in NR6112 and cannot be easily explained by slippage at contiguous bases, direct or inverted repeats or hypothetical DNA secondary structures.

b) Frameshifts at A:T sites. Thirteen frameshift mutations occurred at A:T base-pairs (4 addition, 9 losses). Nine of these mutations occurred in excision repair proficient strains which were

relatively poorly mutated by 1,8-NONP. The ratio of base additions to base losses, as well as the relatively high proportion of these mutations which were recovered from NR6112 and EE125 suggest that some of these mutations might have arisen spontaneously. Twelve out of the 13 mutations recovered at A:T sites occurred in runs of contiguous adenine residues; six of these mutations occurred at the two sites in the lacI gene which contained a run of 5 contiguous A:T base pairs. These observations are consistent with a slippage model for frameshift mutagenesis.

c) -(GC:CG) (-2) Frameshift Mutations. Frameshift mutations resulting from the loss of GC:CG base pairs occurred at 4 different sites. Three of these sites contain alternating GC sequences: positions 250-253 (5'-TGCACGCGCCGT-3'), 575-578 (5'-AATCGCGCTGTT-3'), and 790-795 (5'-CAATGCGCGCCATT-3'). Mutations at these sites as well as at the alternating GC sequence (5'-CTGCCGCGCGCGGACA-3') of the hisD3052 locus of S. typhimurium TA1538 and TA98 might occur as a result of incorporation-slippage as shown in Figure 31. On the basis of modelling studies with AAF modified dodecamers, a variety of B-DNA structures have been proposed which could yield -2 frameshifts due to adduct induced distortion of extended alternating GC sequences (Broyde and Hingerty, 1987). Whether these structures are also applicable to the 1,8-NONP adduct or to the relatively short alternating GC sequences present in the lacI gene is unknown.

Fuchs and coworkers have postulated that B => Z transitions in alternating GC sequences could be important to the formation of -2 frameshift mutations (Fuchs et al., 1988; Freund et al., 1989;

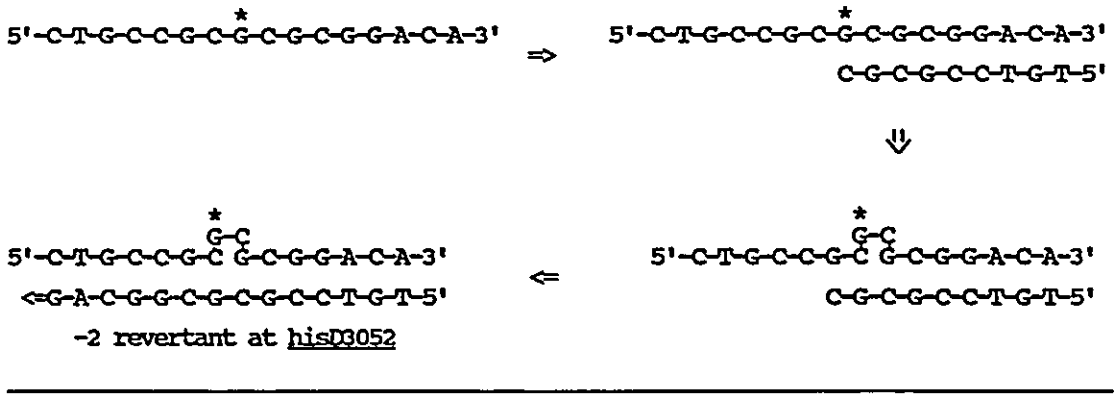


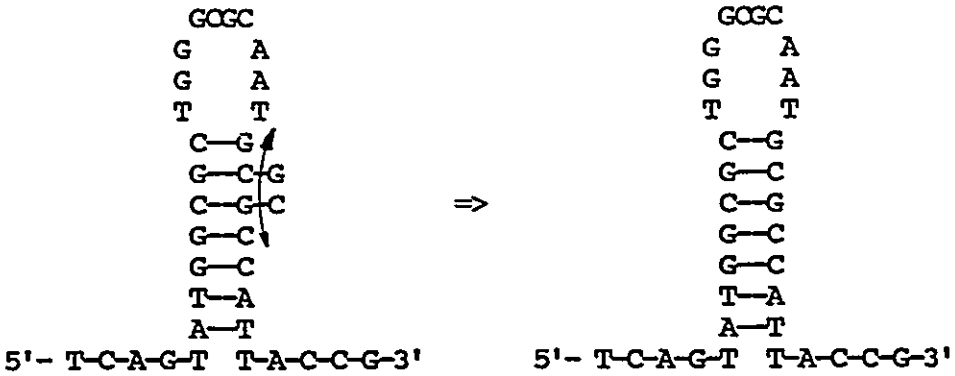
Figure 31. Incorporation-slippage model for -2 frameshift mutations. The framehsift shown here is a -(GC:CG) at the hisD3052 locus of S. typhimurium, and might also be relevant to other alternating GC sequences.

Koffel-Schwartz and Fuchs, 1989), and that AAF might be capable of inducing B => Z transitions in these sequences (Burnouf et al., 1989). DNA conformation might also be important to the -2 frameshift mutations induced by 1,8-NONP or 1,8-DNP. The low number of -2 frameshifts actually recovered following 1,8-NONP treatment might simply be a reflection of the limited ability of the lacI sequences to undergo B => Z transitions.

The -(GC:CG) frameshifts recovered in this study might have been templated by sequences somewhat removed from the site of the mutation. For instance, the -2 frameshift at position 790-795 could be templated by either direct or invert repeats. Figure 32a shows an imperfect palindromic structure which is improved by the loss of a -(GC:CG) sequence. In this case the mutation would be templated by an inverted repeat at positions 773-779. Figure 32b shows that the sequence surrounding positions 790-795 contains a large number of direct repeats with varying degrees of homology to the mutated sequence. Thus, the -2 frameshift at this position might be templated by a direct repeat five bases in length at position 813-817, or direct repeats 4 bases in length at positions 775-778, 783-786, or 823-826.

Ripley et al. (1986) observed that, in the rIIB gene of bacteriophage T_4, a large proportion of -2 frameshifts occurred at sites immediately adjacent to potential palindromes. In order to determine whether this might also be the case for the -(GC:CG) frameshifts recovered following 1,8-NONP treatment, the potential of sequences immediately adjacent to the -(GC:CG) mutation to form

a) -(GC:CG) at 790-795 via a palindromic intermediate



b) -GC:CG) at 790-795 via direct repeats

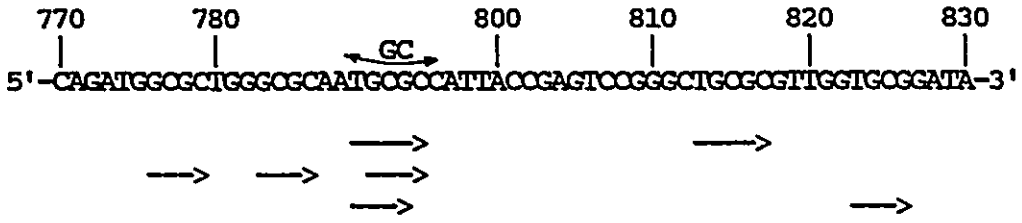


Figure 32. Templating of -(GC:CG) at position 790-795 by means of inverted and direct repeats.

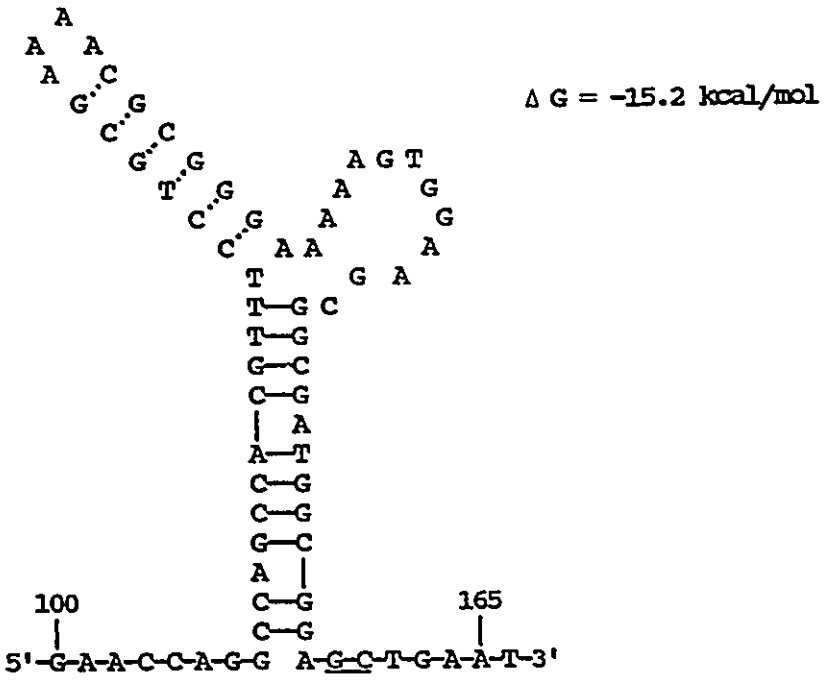
hairpin structures was examined. At two of the four sites possible hairpin structures were obtained. These are shown in Figure 33. The -2 frameshift at position 160-161 is at the base of the structure shown in 33a. The -2 frameshift at position 250-253 is situated at the base of an extremely G:C rich (8/9 base-pairs) hairpin (Figure 33b) containing 2 unpaired bases. Both of these structures are predicted to be stable using the program developed by Zuker and Stiegler (1981). No energetically favorable secondary structures could be found which might account in a similar manner for the -(GC:CG) frameshift mutations at positions 575-578 or 790-795.

The unexpected observation that -2 frameshift mutations occur infrequently in the lacI gene following 1,8-NONP treatment suggests that the DNA sequence requirements for efficient mutagenesis by this pathway are extremely stringent. The above discussion shows that while the low frequency of -2 mutational events recovered in this study might be attributable to relatively inefficient processing of the dG-C(8) adduct through a slippage mechanism, alternate explanations based on DNA conformational changes, DNA secondary structure, or long range misalignment between direct repeats are also possible. In the latter context, the presence of a bulky adduct might cause the DNA polymerase to halt and/or dissociate from the replication fork. This would promote the formation of extensive stretches of single-stranded DNA, and would provide increased time for the formation of DNA secondary structures.

3.4.4 Deletion Mutations

Deletion mutations represent a very small fraction of

a) -GC:CG) at position 160-161 due to adjacent palindrome



b) -(GC:CG) at position 250-253 due to adjacent hairpin structure

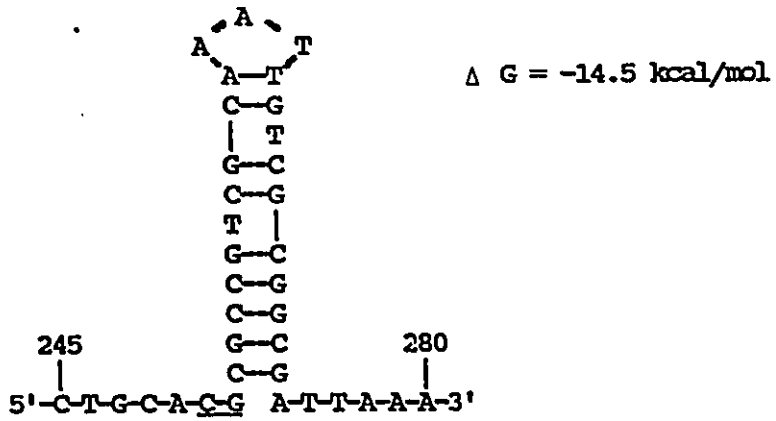


Figure 33. -2 Frameshifts adjacent to palindromes.
The deleted bases at the base of the palindrome are underlined.

mutations induced by 1,8-NONP treatment. Forty deletion mutations were recovered in this study (Table 15). The frequency of deletions was increased markedly by 1,8-NONP treatment in CM6114 (uvrB$^-$, pKM101) (Table 20). In the uvrB$^+$ strains NR6112 and EE125, the frequency of deletion mutations following treatment was very close to the spontaneous level. Only 2 deletion mutations were recovered from the uvrB$^-$ strain NR6113. However, it is likely that the mutants which were selected following 1,8-NONP treatment contain more deletion mutations than is suggested by the number that were actually recovered onto mRS81. As previously discussed (sections 3.3.4 and 3.4.1), this is because deletions were recovered onto mRS81 at lower frequency than point mutations, and consequently are likely to constitute a significant fraction of unrecovered mutations.

This study provides the first evidence that deletion mutation might be an outcome of DNA adduct formation by nitropyrenes. This observation that bulky DNA damage can induce deletion mutations is consistent with a previous report of BPDE-induced (Kokontis et al., 1988) deletion mutations in E. coli. There have also been several reports of UV-induced deletions in the lacI gene (Miller, 1985; Schaaper et al., 1987; LeClerc et al., 1988).

Some deletions were recovered more than once: the 40 deletion mutations which have been sequenced comprised 24 distinct mutational events. The deletion at 267-282 was recovered from 3 of the strains a total of 9 times. Other deletions were also recovered from more than one strain; these include 339-357, 331-350, and 917-969, each of which occurred in 2 strains. Six of the deletion sites

have been detected in previous studies: 143-329 (Schaaper et al., 1987); 147-269 (Farabaugh et al., 1978; Schaaper, et al., 1986; Sedwick et al., 1986); 267-282 (Schaaper et al., 1986; 1987; Gordon et al., 1988b); 301-423 (Fix et al., 1987; Schaaper et al., 1987); 331-350 (Farabaugh et al., 1978; Schaaper et al., 1987; Halliday and Glickman, unpublished results); and 917-969 (Sedwick et al., 1986; Schaaper et al., 1987; Halliday and Glickman, unpublished results). Thus, 18 new deletion sites were characterized in these experiments. The endpoints of 35 deletions contain homologous (≥80 %) sequences of between 4 and 15 bases. Direct repeats have previously been identified at the endpoints of lacI deletions arising either spontaneously (Farabaugh et al., 1978; Albertini et al., 1982; Schaaper et al., 1986; Sedwick et al., 1986; Fix et al., 1987) or following u.v. irradiation (Schaaper et al., 1987).

Both replication and recombination models have been proposed to account for the site specificity of deletion mutations (Singer and Westlye, 1988). These are illustrated in Figure 34. During replication, deletions could result from slippage of the growing primer strand between the two direct repeats on the template strand (Figure 34a). The deleted sequence would be comprised of one copy of the repeat plus the sequence between the two repeats. Subsequent rounds of replication, or excision of the looped out segment by repair endonucleases, would produce a stable deletion mutation. Intrachromosomal recombination (Figure 34b) would also yield a deletion mutation. Both legitimate (Smith, 1988) and illegitimate (Ehrlich, 1989) modes of recombination have been identified in

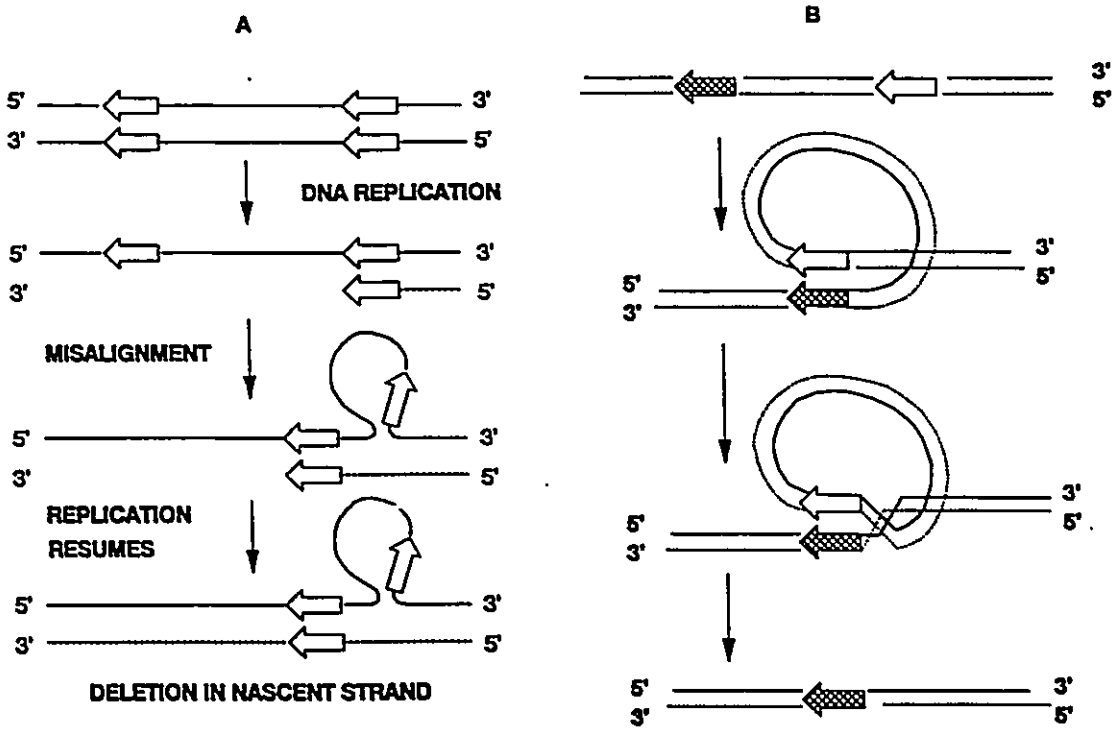


Figure 34. Models for deletion formation.
a) Deletion formation as a result of strand slippage between direct repeats during DNA synthesis. The arrows represent direct repeats. The newly synthesized repeat might melt, and reassociate with the repeat located further downstream. Slippage could be enhanced if the looped out strand contained inverted repeats and formed a palindromic structure. This would bring the repeats closer together and increase the probability that dissociation/reassociation of the nascent strand would create a deletion (Ripley and Glickman, 1983; Glickman and Ripley, 1984).
b) Deletion formation as a result of intrastrand recombination between homologous sequences (arrows).

bacteria. Legitimate recombination could be mediated by either the recBC or recF pathway. The recBC pathway has a minimum requirement for 23-27 base pairs of homology, while the homology requirements for recF mediated recombination are considerably greater (44-90 base pairs) (Shen and Huang, 1986). Although several reports have suggested that deletion formation is recA independent (reviewed in DasGupta et al, 1987), at least one study (Albertini et al., 1982) has suggested that deletion frequency might be enhanced by functional RecA protein. Illegitimate recombination is very poorly understood. Extensive homolgies are not observed at putative sites of illegitimate recombination, although short (3 to 20 base pairs) sequences of homology are common (Ehrlich, 1989). Enzymes which break and/or join DNA such as DNA gyrase (Marvo et al., 1983; Ikeda et al., 1984), or MutH protein (Lahue et al., 1989) might be important to recombination by this pathway (Singer and Westlye, 1988; Ehrlich, 1989).

It has been suggested that palindromic structures within the deleted sequence might play a role in deletion formation by bringing the deletion endpoints more closely together (Glickman and Ripley, 1984; Schaaper et al., 1986), or by slowing the progression of replication, thus allowing more time for slippage to occur (Weston-Hafer and Berg, 1989). Although palindromic structures are found among the deletions recovered following 1,8-NONP treatment (for example, Figure 35), palindromy within the deleted sequence is not common in this collection of deletions.

It has previously been suggested that the frequency of

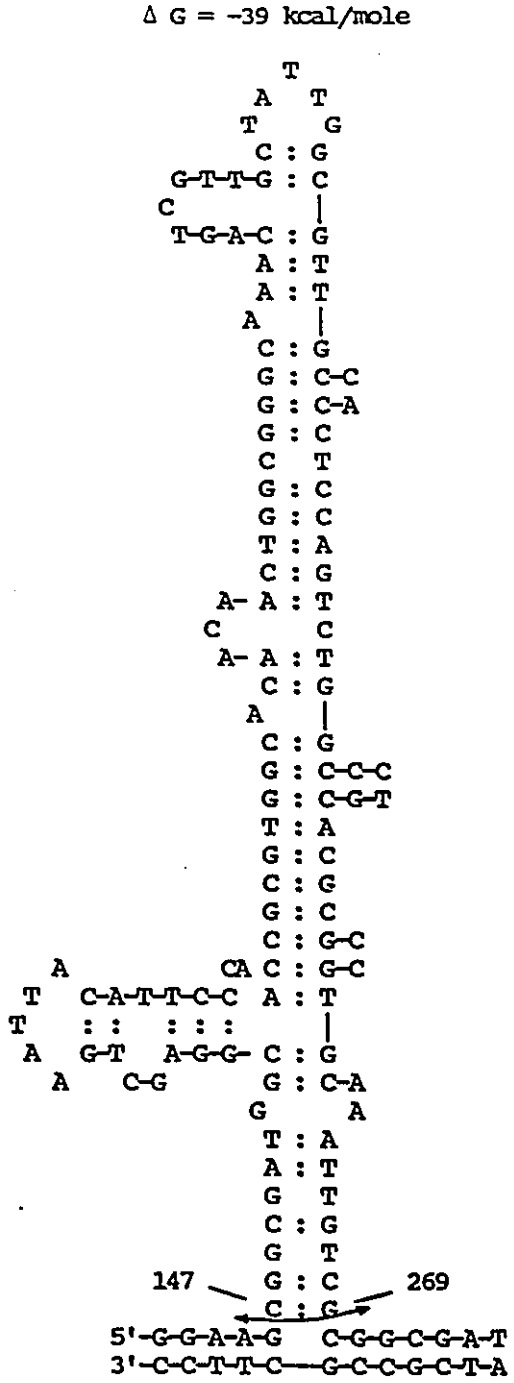


Figure 35. Structural intermediates for deletion formation. This palindrome predicts the exact endpoints for the deletion (147-269) which was recovered three times in this study. This structure was initially proposed by Glickman and Ripley (1984).

deletions increases when the repeats are rich in G:C base pairs (Singer and Westlye, 1988). To evaluate whether there was any difference in G:C richness between sequences on either side of the endpoints of deletion mutations recovered following 1,8-NONP treatment, the number of G:C residues was determined within the first 7 base pairs on either side of the 3'-endpoint of each of the 35 deletions containing repeats. On the side of the endpoint containing the repeat (or the majority of the repeat), 73 % of the sequence was comprised of G:C residues; on the other side of the endpoint there were an average of 50 % G:C base pairs (the average G:C richness of the lacI gene is 56 %). In the context of the mechanism of 1,8-NONP-induced mutation, this apparent bias might be explained by increased levels of DNA adduct formation within G:C rich versus A:T rich sequences. As previously discussed, DNA adduct formation might stall replication, allowing more time for misalignment to occur. When blockage of DNA synthesis occurs within repeats, the misalignment could yield deletions. The relatively large 1,8-NONP-induced increase in deletion frequency which were observed only in CM6114 (pKM101, uvrB$^-$) might reflect the greater efficiency with which DNA synthesis resumes following slippage in the presence of the plasmid encoded functions.

The endpoints of many deletions contain sequences which might be nicked by enzymes involved in DNA replication and repair. For instance, the 3'-endpoint of the deletion 351-394 (which does not contain direct repeats), is at a 5'-GATC-3' sequence; this sequence is the site of the MutH endonuclease activity during methyl-directed

mismatch repair (Lahue et al., 1989). Similarly, the endpoints of several of the deletions contain sequences which are very similar to (though not identical to) the DNA gyrase consensus sequence (5'-RT*GRYC(T/G)Y-3' (* is the gyrase cut site) (Ehrlich, 1989)). An example of this is the deletion -42-357 which does not have direct repeats at its endpoints: the sequences 5'-AT*GGTGTC-3' (transcribed strand) and 5'-AT*GGTTGC-3' (nontranscribed strand) are on either side of the 5'-endpoint of this deletion. Each of these sequences differs from the gyrase consensus sequence by only a single base. The presence of such sequences at deletion endpoints is consistent with the notion that deletion formation can be stimulated by errors in DNA breakage-reunion reactions. Whether bulky DNA adducts can increase the frequency of these errors is a question that requires much more experimental data.

3.4.5 Other mutations

A number of mutations other than base substitutions, frameshifts, and deletions were recovered following 1,8-NONP treatment. These include 193 +TGGC mutations and 63 -TGGC mutations, 5 duplications, 3 complex mutations and 2 tandem base substitutions.

Hotspot mutations (+TGGC and -TGGC) were recovered from all strains treated with 1,8-NONP. However, all but 25 (19 +TGGC and 6 -TGGC) of these mutations were recovered in the uvrB$^+$ strains NR6112 and EE125. Since hotspot mutations constitute almost 70% of all spontaneous mutations in the lacI gene, it is likely that the majority of the hotspot mutations recovered following 1,8-NONP treatment were also of spontaneous origin. However, it is possible

that the presence of increased levels of DNA adducts might stall replication and/or destabilize the helix to such an extent that a slight increase in the frequency of some spontaneous mutations would result. This would be an example of untargeted, or semitargeted mutagenesis (Schaaper et al., 1987).

Only 5 duplications at 4 sites were recovered following 1,8-NONP treatment. Two of the sites contain direct repeats at the endpoints. Duplication 613-618 (found twice) has the sequence 5'-GCGTCTG-3' at each endpoint, and is also immediately adjacent to the site of +TGGC and -TGGC mutations (5'-TGGCTGGCTGGC-3' at position 621-632). Duplication 695-714 contains the sequence 5'-CCATG-3' at each endpoint. The observation that duplication endpoints contain homologous sequences has been reported previously (Edlund and Nordmark, 1981), and is consistent with a slippage mechanism for duplication formation. Two of the duplications, at 345-505 and 863-883, do not contain direct repeats or any other structural feature which would suggest their origin.

3.4.6 Summary

The spectrum of mutations recovered from bacteria treated with 1,8-NONP illustrates how the nature of the local DNA sequence can influence the process of mutagenesis. A variety of mechanisms have been proposed which are consistent with the observed mutational specificity. These mechanisms emphasize different outcomes of replication across from bulky DNA adducts. An important intermediate in the proposed mechanisms is a stalled DNA polymerase at the site of the adduct. Resolution of this intermediate is likely to be

modulated by the nature of the surrounding sequence, and the activity of cellular error-prone repair functions. At least three types of mutation might be derived through such an intermediate: slippage of the newly synthesized strand either one base, or several bases, would be stabilized by sequences complementary to the newly synthesized primer strand, and would result in frameshift mutations and deletions, respectively; incorporation of a wrong base without slippage would yield a base substitution mutation if the necessary cellular functions were present to allow resumption of replication.

A number of other factors are likely to influence the formation of mutations at a particular site. These include the relative reactivity of bases within certain sequences; the ability of sequences to stabilize extrahelical structures (both single bases and large palindromes); the preference of base incorporation by DNA polymerases; the susceptibility of bases incorporated opposite DNA adducts to repair by the exonucleolytic (proofreading) activity of DNA polymerase, or the mismatch repair system; and the influence (if any) of DNA adducts on the accuracy of enzymes which cleave and anneal DNA.

4. CONCLUSION

4.1 Summary

Nitropyrenes were initially identified as potential human health hazards as a result of their potent, direct-acting mutagenicity in bacteria (Lofroth et al., 1980; Rosenkranz et al., 1980). Since that time numerous studies have investigated the metabolism of these compounds; the DNA adducts formed; and their mutagenicity in bacterial reversion assays (reviewed in Chapter 1; Rosenkranz and Mermelstein, 1983; Tokiwa and Ohnishi, 1986). However, previous experiments have provided limited information regarding the specificity of nitropyrene-induced mutation. The results of the experiments reported in this thesis have directly addressed the issue of mutational specificity. This data, in conjunction with knowledge of the DNA adducts formed by 1,8-NONP, provides insight regarding potential mechanisms of mutation. On the basis of these studies, several conclusions can be drawn regarding the action of 1,8-NONP in E. coli. The following points have been discussed in detail in the main text of the thesis.

1) 1,8-NONP forms a single major DNA adduct, and two minor adducts. The major adduct is formed by reaction of the nitrenium ion derived from 1,8-HANP with the C(8) position of dG. One of the minor adducts probably results from reaction with dA, while the other is a product of dG or dC.

2) DNA adducts formed with 1,8-NONP are efficiently recognized and repaired by the uvrABC eximuclease. This results in an

extremely low induced mutation frequency in E. coli strains which are proficient in excision repair.

3) 1,8-NONP induces at least 3 different classes of mutation in the lacI gene of E. coli: frameshift mutations, base substitutions, and deletions.

4) Frameshift mutations occur primarily at G:C base pairs. It is likely that the frameshifts are targeted by the major dG-C(8)-ANP adduct; however, the possibility that the minor dG or dC adduct plays a role in frameshift mutagenesis cannot be entirely ruled out. The distinctive specificity of 1,8-NONP-induced frameshifts is consistent with an "incorporation-slippage" model for frameshift mutation. The sites at which frameshifts occur suggests that some of these mutations might also be templated by direct or inverted repeats within flanking DNA sequences.

5) Base substitutions are also targeted events: while most of these mutations appear to be targeted by dG-C(8)-ANP, a small number are likely to occur as the result of adduct formation on dA. Base substitution mutation is strongly influenced by the presence of cellular error-prone repair functions. The most prominent base substitution mutations are G:C => T:A transversions; this is consistent with the notion that bulky DNA lesions are bypassed during DNA synthesis with the incorporation of adenine.

6) The endpoints of deletion mutations contain directly repeated DNA sequences. These data are most consistent with a slippage model for deletion mutation. In some cases it is possible that the formation of palindromic structures facilitates formation of

the deletion intermediate. Deletion mutation might also be promoted by the activity of enzymes which nick DNA during replication and repair.

4.2 Genotoxicity of Nitropyrenes in Mammalian Cells

There are practical reasons for studying mutational mechanisms. The observation that many mutagens induce tumors in mammals (Ames, 1979) implies that DNA is a principal target for many carcinogens. An important goal of mutation research is to understand the mechanisms through which chemicals interact with critical cellular targets to promote tumor development, and to devise appropriate strategies for detecting carcinogens. At the present time bacterial mutagenicity assays are part of the battery of tests which are used to evaluate whether individual chemicals represent a potential human health hazard. The results of such assays are likely to be particularly relevant when a chemical acts in a similar manner in bacterial and mammalian cells. Several lines of evidence suggest that studies of the action of nitropyrene derivatives in bacteria provide information which is pertinent to their genotoxicity in mammalian cells.

1) In E. coli, three DNA adducts are formed by 1,8-NONP. All of these adducts are produced by the highly reactive nitrenium ion (or resonance stabilized carbenium cation) derived from 1,8-HANP. ^{32}P-Postlabelling studies in our laboratory have shown that precisely the same pattern of adducts was observed in rabbit tracheal epithelial cells which had been treated with either 1,8-NONP or 1,8-DNP (Norman et al., 1989a). In addition, dG-C(8)-ANP was the major

DNA adduct detected in rat mammary, mesentery, bladder, lung, kidney and liver following an i.p. injection with 1,8-DNP (Norman, 1988). These results suggest that the reactive intermediates which result from reductive metabolism of 1,8-DNP in mammalian cells, and the types of DNA lesions produced, are qualitatively similar to those which are produced in E. coli by treatment with 1,8-NONP. Studies in other laboratories have shown that a dG-C(8) product is also the principal DNA adduct formed in rodents following administration of radiolabelled 1,6-DNP (Delclos et al., 1987a; Djuric et al., 1988).

2) The tissue distribution of DNA adducts resulting from an i.p. injection of 1,8-DNP has been examined in female CD rats (Norman, 1988). Twelve hours after treatment, the levels of dG-C(8)-ANP in the mammary gland and mesentery tissue were shown to be between 4 and 15-fold higher than in liver, kidney, lung, or bladder. In addition, studies monitoring the kinetics of dG-C(8)-ANP removal suggested that, in the mammary gland, this DNA adduct was relatively more persistent than in other tissues (Norman, 1988). These results correlate with the major sites of tumor formation following i.p. injection of 1,8-DNP, which are the mammary tissue (mammary adenocarcinoma and fibroadenoma) and the peritoneal cavity (malignant fibrous histiocytoma) (King, 1988).

3) Maher and coworkers have examined the mutational specificty of several compounds in mammalian cells using a shuttle vector containing the supF gene as a mutational target. G:C => T:A transversions were the most common mutation recovered in the supF gene when a 1-NOP-modified shuttle vector was allowed to replicate in

human cells (Yang et al., 1989). This correlates well with the specificity of 1,8-NONP-induced base substitution in the lacI gene of E. coli.

4) The ras family of oncogenes are known to be activated by point mutations in codons 12, 13, or 61 (Guerrero and Pellcier, 1987). Activated ras genes have been detected in many different tumors obtained from experimental animals (Guerrero and Pellcier, 1987) and humans (Bos, 1989). Ochai et al. (1985) showed that a fibrosarcoma induced by 1,8-DNP contained an activated K-ras gene; the activating mutation was shown to be a G:C => T:A transversion at codon 12 (Tahira et al., 1986). This is consistent with the most common type of base substitution mutation recovered in E. coli following 1,8-NONP treatment.

4.3 The Analogous Biological Activity of Nitropyrenes and Aromatic Amines

As discussed in Chapter 2, nitropyrenes and aromatic amines are very similar in certain aspects of their metabolism (i.e. N-hydroxy arylamine derivatives are common metabolic intermediates) (King, 1988), and in the nature of the DNA adducts which are formed (Beland et al., 1983; Beland and Kadlubar, 1985; Andrews et al., 1986; Norman, 1988; Norman et al., 1989a). The studies reported in this thesis, along with several studies which have examined the mutational specificity of arylamine derivatives, provide evidence that the dG-C(8) adducts formed by nitropyrenes also produce mutations similar to those induced by arylamines in bacteria. In E. coli, frameshift mutations and G:C => T:A transversions are the most

common mutations induced by the aromatic amine derivatives AAF (Miller, 1983; Koffel-Schwartz et al., 1984) and AF (Bichara and Fuchs, 1985), as well as the nitropyrene derivative 1,8-NONP.

There is also evidence that nitropyrenes and aromatic amines have similar effects in mammalian cells. In cultured human 293 cells, the dG-C(8) adducts produced by either AF or 1-NOP yielded the same type of mutation, G:C => T:A transversions, in the supF gene (Yang et al., 1989). In CHO cells the level of dG-C(8) adduct formation by AF, N-hydroxy-N'-acetyl-benzidine and 1-NOP each correlated strongly with the induction of gene mutations and sister chromatid exchanges (Heflich et al., 1986). G:C => T:A transversions have been detected in activated ras oncogenes recovered from tumors induced by either AAF (Wiseman et al., 1986), or 1,8-DNP (Ochai et al., 1985; Tahira et al., 1986). In addition, it is well established that both nitropyrenes (section 1.1.3) and aromatic amines (Clayson and Garner, 1976) are capable of inducing tumors in experimental animals. The striking similaties between the two groups of chemicals have important implications since aromatic amines are recognized to be human carcinogens (Clayson and Garner, 1976; Parkes, 1976; King, 1988).

4.4 Concluding Remarks

Studies of mutagenesis in S. typhimurium and E. coli have been central to our present understanding of the mechanisms by which mutagens interact with DNA to produce heritable changes within the genome (Drake and Baltz, 1976; Friedberg, 1985; Eisenstadt, 1987). These bacteria, and their phages, have proven to be particularly well suited to genetic analysis. Moreover, the availability of well

characterized mutants (defective in specific cellular functions such as DNA repair, recombination etc.) has allowed workers to evaluate how cellular processes influence mutagenesis. We know much less about the mechanisms of mammalian cell mutation. The analysis of mutation in mammalian cells is slower and more cumbersome than in bacteria, and is hindered significantly by a lack of knowledge regarding the influence of cellular factors in mutagenesis. The recent development of techniques which allow forward mutations to be cloned and sequenced has made it possible to determine the specificity of spontaneous or induced mutation in both prokaryotic and eukaryotic cells. The direct comparison of the mutational specificity of mutagens in different organisms will provide future opportunities to evaluate the similarities and differences between the mechanism of action in these organisms.

To date, a variety of experimental systems have been used to examine the mutational specificity of a relatively small number of prototypic mutagens. It is likely that additional information can be obtained from studies which use a single mutational target to examine the specificity of several different mutagens. In this context, the data presented in this thesis provide a detailed description of the mutations induced by a mutagen (1,8-NONP) which has not previously been studied at the DNA sequence level. Since the *lacI* gene is currently used in several laboratories for mutation studies in both bacteria (Gordon *et al.*, 1988a) and mammalian cells (DuBridge and Calos, 1988), the 1,8-NONP data contribute to an expanding and important data base.

Appendix 1

The following table shows the results of the genetic, oligonucleotide probing, and DNA sequencing experiments which characterized the lacI⁻ mutations recovered following 1,8-NONP treatment of E. coli strains NR6112, EE125, NR6113, and CM6114. Those mutants which contained +TGGC or -TGGC mutations are not included.

Legend

CSH52: The colour of CSH52(F'lacI⁻) sexductants on XGal plates- B, Dark blue (Dominant mutations); b, light blue; w, slightly blue; W, White (recessive mutations).

Su3, SuB, SuC: The suppressibility of lacI⁻ nonsense mutations by glutamine (SuB) and tyrosine (Su3, SuC)- W, white (suppressed); w, slightly blue; b, light blue; B, Dark blue (not suppressed).

Probing: The results of oligonucleotide probing studies. WT: the wild-type probe (position 617-636) hybridizes to DNA obtained from lacI⁻ mutants. MINUS: None of the three oligonucleotide probes (wild-type, +TGGC, or -TGGC) hybridizes with lacI⁻ DNA.

Mapping: The results of deletion mapping. The primer which was used to initiate DNA sequencing is shown. P1, position 148-161; P2, 302-315; P3, 450-463; P4, 604-617; P5, 745-758; P6, 901-914; P7, 1049-1062; PL, universal primer. Mutants which were dominant in CSH52, and mapped to P1/P2 were sequenced with primer P1C, position 215-228.

Designation: DNA sequencing results. For frameshift mutations the position denoted is the most 3' in a contiguous run. NC, not characterized; NR, not recovered.

I.1) NR6112 Mutations

Mutant	CSH52	Su3	SuB	SuC	Probing	Mapping	Designation
K2	W	B	B	B	MINUS	P3	Deletion (?-974)
K3	b	W	B	B	WT	P1/P2	C(104) => T
K4	B	B	B	B	WT	P1/P2	-A(190)
K6	B	B	B	B	WT	PL	NR
K8	W	B	B	B	WT	P6	Duplication (695-714)
K9	W	B	B	B	WT	P4	-G(525)
K10	W	B	B	B	WT	P1/P2	NR
K11	w	B	B	B	WT	P1/P2	Deletion (147-269)
K12	W	B	B	B	WT	P4	-G(577C578)
K17	W	B	B	B	WT	P6	-G(843)
K19	b	B	B	B	WT	P1/P2	G(111) => A
K21	W	B	B	B	MINUS	P3	Deletion (454-796)
K22	B	B	B	B	WT	P6	NR
K24	B	B	B	B	WT	P1/P2	-A(190)
K25	W	B	B	B	WT	P5	NR
K26	W	B	W	W	WT	P4	G(437) => T
K28	b	B	B	B	WT	P1/P2	Deletion (147-269)
K29	W	B	B	B	WT	P3	Deletion (351-394)
K31	W	B	B	B	WT	P3	-C(485)
K33	B	B	B	B	WT	P1/P2	C(186) => A
K39	w	B	B	B	WT	P3	NC
K46	W	B	B	B	WT	P3	Deletion (331-350)
K49	B	B	B	B	WT	P1/P2	-A(190)
K51	W	B	B	B	WT	P4	-C(493)
K53	W	B	B	B	WT	P6	Deletion (917-969)
K56	W	B	B	B	WT	P4	Deletion (418-425)
K57	B	B	B	B	WT	P1/P2	-C(175)
K59	b	B	B	B	WT	P1/P2	Deletion (-88-78)
K60	W	B	B	B	WT	P6	-G(919)
K64	W	B	B	B	WT	P1/P2	G(222) => A
K65	B	B	B	B	WT	P6	NR
K71	B	W	W	W	WT	P1/P2	C(210) => A
K74	W	B	B	B	WT	P6	-G(972)
K80	B	B	B	B	WT	P6	-C(745)
K84	W	B	B	B	WT	P6	-G(843)
K92	W	B	B	B	WT	P6	G(783) => A
K102	W	B	B	B	WT	P1/P2	NC
K112	W	B	B	B	WT	P5	+C(604)
K113	W	B	B	B	WT	P1/P2	-C(252G253)
K115	W	B	B	B	WT	PL	-A(1010)
K121	B	B	B	B	WT	P1/P2	G(180) => C
K135	B	B	B	B	WT	P1/P2	C(84) => A
K136	W	B	B	B	WT	P6	-G(843)
K140	W	B	B	B	WT	P6	NC
K143	W	B	B	B	WT	P1/P2	-G(160)C(161)
K148	B	B	B	B	WT	PL	NR
K150	W	B	B	B	WT	P1/P2	Deletion (267-282)
K151	W	B	B	B	WT	P6	NR
K153	W	B	B	B	WT	P3	Deletion (339-357)

Mutant	CSH52	Su3	SuB	SuC	Probing	Mapping	Designation
K158	W	B	B	B	WT	P1/P2	A(189)AC => GCCC
K159	W	B	B	B	WT	P5	-G(783)
K160	B	B	B	B	WT	P5	NR
K166	W	B	B	B	WT	P6	Deletion (917-969)
K172	w	B	B	B	WT	P1/P2	-A(139)
K173	W	B	B	B	WT	P5	-C(591)
K174	W	B	B	B	WT	P6	-C(988)
K175	W	B	B	B	WT	P6	-G(811)
K176	W	B	B	B	WT	P6	-A(898)
K177	W	B	B	B	WT	P1/P2	-C(229)
K181	W	B	B	B	WT	P6	-G(919)
K183	W	B	B	B	WT	P5	-C(485)/A(483) => G
K184	W	B	B	B	WT	P3	Deletion (306-316)
K185	B	B	B	B	WT	P1/P2	-C(92)
K187	B	B	B	B	WT	P1/P2	-C(175)
K188	W	B	B	B	WT	P6	Deletion (776-815)
K191	W	B	B	B	WT	P5	Duplication (613-618)
K193	W	B	B	B	WT	P1/P2	-G(222)
K194	W	B	B	B	WT	P5	-G(561)
K195	W	B	B	B	WT	P1/P2	+C(92)
K199	W	B	B	B	WT	P6	-G(843)
K201	W	B	B	B	WT	P3	-G(413)
K203	W	B	B	B	WT	P1/P2	NR
K205	W	B	B	B	WT	P1/P2	-G(222)
K207	W	B	B	B	WT	P1/P2	Deletion (267-282)
K209	W	B	w	b	WT	P6	C(867) =>A
K210	W	B	B	B	WT	P6	Deletion (788-796)
K213	W	B	B	B	WT	P6	NC
K214	W	B	B	B	WT	P1/P2	Deletion (267-282)
K217	B	B	B	B	WT	P1/P2	G(93) => A
K218	W	B	B	B	WT	P6	Deletion (943-969)
K219	W	B	B	B	WT	P1/P2	Deletion (301-423)
K225	W	B	B	B	WT	P1/P2	Deletion (301-423)
K228	W	B	B	B	MINUS	P1/P2	NR
K239	w	B	B	B	WT	P1/P2	Deletion (147-269)
K244	W	B	B	B	WT	P7	-G(919)
K245	B	B	B	B	WT	P1/P2	-G(242)
K247	W	B	B	B	WT	P1/P2	Deletion (267-282)
K248	B	B	B	B	WT	P1/P2	G(65)C(66) =>AA
K252	B	B	B	B	WT	P1/P2	-C(175)
K257	W	B	B	B	WT	P5	-G(588)
K258	W	B	B	B	WT	P6	-G(783)
K260	W	B	B	B	WT	P6	NC
K262	W	B	B	B	WT	P5	-G(811)

I.2) EE125 Mutations

Mutant	CSH52	Su3	SuB	SuC	Probing	Mapping	Designation
L3	W	nd	B	nd	WT	P6	NC
L8	w	B	W	W	WT	P1/P2	C(49) => A
L9	W	B	B	B	WT	PL	NC
L10	W	B	B	B	WT	P7	C(928) => A
L11	W	B	B	B	WT	P4	-G(515)
L12	W	W	B	B	WT	P6	G(803) => T
L13	W	B	B	B	MINUS	P5	G(623) => T
L14	B	B	B	B	WT	P1/P2	C(150) => A
L15	W	B	B	B	WT	P6	A(1013) => C
L16	W	B	W	W	WT	P7	C(993) => A
L17	B	B	B	B	WT	P1/P2	+G(134)
L18	W	B	B	B	WT	P7	-G(843)
L19	W	B	B	B	WT	P6	NC
L21	W	B	B	B	WT	P7	NC
L22	W	B	B	B	WT	P6	-G(927)
L25	W	B	B	B	WT	P1/P2	-C(245)
L26	W	W	b	b	WT	P3	NR
L27	W	B	B	B	WT	P5	-G(677)
L31	W	B	B	B	WT	PL	NC
L32	W	B	B	B	WT	P1/P2	G(487) => T
L33	W	B	B	B	WT	P1/P2	-C(245)
L34	W	B	B	B	WT	P6	NC
L36	W	B	B	B	WT	P4	-G(525)
L37	B	B	B	B	WT	P1/P2	T54 => G
L39	B	B	B	B	WT	P1/P2	T72 => A
L40	W	B	B	B	MINUS	P1/P2	NR
L45	W	B	B	B	WT	P5	C(693) => A
L46	W	B	B	B	WT	P1/P2	+A(187)
L47	W	B	B	B	WT	P6	C(834) => A
L48	W	B	B	B	MINUS	P1/P2	NR
L50	W	B	W	W	WT	P4	NC
L54	W	B	B	B	WT	P6	NC
L56	b	B	B	B	WT	P6	NR
L59	B	B	B	B	WT	P1/P2	G(107) => A
L60	B	B	B	B	WT	P1/P2	C(57) => A
L63	W	B	B	B	WT	P6	G(842) => T
L70	W	B	B	B	WT	P5	Duplication (613-618)
L73	W	B	B	B	WT	P1/P2	Deletion (143-329)
L75	W	B	B	B	WT	P6	NC
L80	W	B	B	B	WT	P1/P2	NR
L83	B	B	B	B	WT	P1/P2	C(90) => A
L84	W	B	B	B	WT	PL	NR
L88	B	B	B	B	WT	P1/P2	T54 => C
L89	W	B	B	B	WT	P6	NC
L90	B	B	B	B	WT	P6	NR
L95	W	B	B	B	WT	P3	-C(385)
L96	W	B	B	B	WT	P1/P2	Deletion (267-282)
L99	B	B	B	B	WT	P1/P2	G(53) => T
L100	B	B	B	B	WT	P5	NR
L102	W	B	B	B	WT	P3	Deletion (331-350)
L105	B	B	B	B	WT	P1/P2	G(93) => T

Mutant	CSH52	Su3	SuB	SuC	Probing	Mapping	Designation
L106	W	B	B	B	WT	P3	-C(427)
L107	W	B	B	B	WT	P3	-C(391)
L108	B	B	B	B	WT	P1/P2	G(53) => T
L109	W	B	B	B	WT	P6	NC
L110	B	B	B	B	WT	P1/P2	-C(92)
L111	W	B	W	W	WT	PL	C(993) => A
L112	W	B	W	W	WT	P6	G(857) => T
L115	W	B	B	B	WT	P1/P2	C(228) => A
L117	W	B	W	W	WT	P3	G(326) => T
L118	B	B	B	B	WT	P1/P2	C(57) => A
L120	b	b	b	b	WT	P1/P2	G(158) => T
L121	W	B	W	W	WT	P7	C(867) => A
L122	W	B	B	B	WT	PL	NC
L123	W	B	B	B	WT	P1/P2	-G(242)
L124	B	B	B	B	WT	P1/P2	G(116) => T
L125	B	B	B	B	WT	P1/P2	G(93) => T
L126	W	B	B	B	WT	P1/P2	C(228) => A
L127	B	B	B	B	WT	P1/P2	-C(92)
L128	W	B	B	B	WT	P1/P2	Deletion (250-353)
L129	W	B	B	B	WT	P7	-A(1010)
L130	B	B	B	B	WT	P1/P2	G(185) => A
L131	W	B	B	B	WT	P4	-C(493)
L132	W	B	B	B	WT	P6	G(1005) => T
L133	W	W	W	W	WT	P6	G(731) => T
L134	B	B	B	B	WT	P1/P2	G(185) => C
L135	B	B	B	B	WT	P1/P2	G(93) => T
L137	W	B	B	B	WT	P6	G(783) => T
L138	b	W	b	b	WT	P1/P2	G(158) => T
L139	W	B	B	B	WT	P1/P2	Deletion (250-353)
L140	B	B	B	B	WT	P1/P2	-C(92)
L141	B	W	b	b	WT	P1/P2	C(104) => T
L142	W	B	B	B	WT	P7	-A(1010)
L143	W	B	B	B	WT	P6	G(842) => T
L145	W	B	B	B	WT	P3	-C(391)
L146	B	B	B	B	WT	P1/P2	-C(92)
L148	W	B	B	B	WT	P5	-C(591)
L149	W	B	B	B	WT	P6	-C(745)

I.3) NR6113 Mutations

Mutant	CSH52	Su3	SuB	SuC	Probing	Mapping	Designation
M1	W	B	B	B	WT	P5	-C(694)
M2	w	B	B	B	MINUS	P4	NR
M3	W	B	B	B	WT	P6	-G(735)
M4	W	B	B	B	WT	PL	NC
M5	W	B	B	B	WT	P1/P2	Duplication (345-505)
M6	W	B	B	B	WT	P5	-G(677)
M7	W	B	B	B	WT	P3	-G(422)
M8	W	B	B	B	WT	P1/P2	-C(235)
M9	B	B	B	B	WT	P1/P2	-C(92)
M10	W	B	W	W	WT	P1/P2	C(260) => T
M11	W	B	B	B	WT	P6	-G(794C795)
M12	W	B	B	B	WT	P1/P2	-C(245)
M13	B	B	B	B	WT	P1/P2	-C(175)
M14	B	B	B	B	WT	PL	NR
M15	W	B	B	B	WT	P3	-C(235)
M16	W	B	B	B	WT	P6	-G(919)
M18	B	B	B	B	WT	P1/P2	-C(92)
M19	W	B	B	B	WT	P3	-G(413)
M20	W	B	B	B	WT	P6	G(842) => A
M21	W	B	B	B	WT	P6	-G(735)
M22	W	B	B	B	WT	P6	-G(851)
M23	W	B	B	B	WT	P6	-G(783)
M24	W	B	B	B	WT	P1/P2	Deletion (-42-357)
M25	W	B	B	B	WT	P7	-G(972)
M26	W	B	B	B	WT	P4	-G(422)
M27	W	B	B	B	WT	P7	-G(957)
M28	W	B	B	B	WT	P4	-C(515)
M29	W	B	B	B	WT	P6	G(782) => T
M30	W	B	B	B	WT	P7	-G(972)
M32	W	B	B	B	WT	P6	-G(919)
M33	W	B	B	B	WT	P5	-G(664)
M34	b	B	B	B	WT	P1/P2	-G(143)
M35	B	B	B	B	WT	P1/P2	C(84) => A
M36	W	B	B	B	WT	P4	-G(588)
M37	W	B	B	B	WT	P7	-G(919)
M38	b	B	B	B	WT	P1/P2	-C(109)
M39	W	B	B	B	WT	P3	-G(413)
M40	W	B	B	B	WT	P4	-C(459)
M41	B	B	B	B	WT	P1/P2	-C(92)
M42	B	W	B	B	WT	P1/P2	C(104) => T
M43	W	B	B	B	WT	P4	-G(588)
M44	W	B	B	B	WT	P3	-G(391)
M45	W	B	B	B	WT	P7	-G(919)
M46	W	B	B	B	WT	P6	G(776) => C
M47	W	B	B	B	WT	P5	-G(677)
M48	W	B	B	B	WT	P4	-C(493)
M49	W	B	B	B	WT	P7	-G(919)
M50	W	B	B	B	WT	P6	-G(811)
M51	W	B	B	B	WT	P1/P2	-C(245)
M52	W	B	B	B	WT	P7	-G(919)

Mutant	CSH52	Su3	SuB	SuC	Probing	Mapping	Designation
M53	B	B	B	B	WT	P1/P2	-G(149)
M54	W	B	B	B	WT	P1/P2	-C(235)
M55	W	B	B	B	WT	P7	-C(493)
M56	W	B	B	B	WT	PL	-G(927)/-G(932)
M57	W	B	B	B	WT	P5	-G(677)
M59	B	B	B	B	WT	P1/P2	-C(92)
M60	W	B	B	B	WT	P1/P2	Deletion (272-377)
M61	W	B	B	B	WT	P4	-C(493)
M62	W	B	B	B	WT	P1/P2	+A(139)
M63	W	B	B	B	WT	P4	-G(413)
M65	W	B	B	B	WT	P6	-G(919)
M66	W	B	B	B	WT	P6	-G(811)
M67	W	B	B	B	WT	P4	-C(493)
M68	W	B	B	B	WT	P1/P2	-C(235)
M70	B	B	B	B	WT	P1/P2	-C(113)
M71	W	B	nd	nd	WT	P5	-G(677)
M72	W	B	B	B	WT	P6	-G(751)
M73	B	B	B	B	WT	PL	NR
M74	W	B	B	B	WT	P3	-C(493)
M75	W	B	B	B	WT	P5	G(702) => A
M76	W	B	B	B	WT	P6	-G(795C795)
M77	W	B	B	B	WT	P7	-G(919)
M80	W	B	B	B	WT	P6	-G(843)
M81	B	B	B	B	WT	PL	NC
M82	W	B	B	B	WT	P4	-G(473)
M83	W	B	B	B	WT	P1/P2	-G(300)
M86	W	B	B	B	WT	P1/P2	-C(304)
M87	W	B	B	B	WT	P1/P2	-C(300)
M88	B	B	B	B	WT	P1/P2	-C(92)
M89	W	B	B	B	WT	P5	-G(919)
M90	W	B	B	B	WT	P3	-G(413)
M91	B	B	B	B	WT	P1/P2	-C(92)
M92	W	B	B	B	WT	P6	-G(783)
M93	B	B	B	B	WT	P1/P2	-G(149)
M95	B	B	B	B	WT	P1/P2	-C(92)
M96	W	B	B	B	WT	P5	-G(688)
M97	W	B	B	B	WT	P6	-G(783)
M98	B	B	B	B	WT	P1/P2	-G(201)
M99	W	B	B	B	WT	P1/P2	-C(245)
M100	W	B	B	B	WT	P3	-G(413)
M101	W	B	B	B	WT	P4	-C(537)
M103	W	B	B	B	WT	P6	-G(919)
M104	W	B	B	B	WT	P5	-G(688)
M105	W	B	B	B	WT	P6	-C(879)
M106	B	B	B	B	WT	P1/P2	-C(92)
M107	W	B	B	B	WT	P5	-G(677)
M108	W	B	B	B	WT	P5	-G(783)
M109	W	B	B	B	WT	P6	-C(879)
M110	W	B	B	B	WT	P6	-C(988)
M111	W	B	B	B	WT	P1/P2	-G(391)
M113	B	B	B	B	WT	P1/P2	G(116) => T
M114	B	B	B	B	WT	P1/P2	-G(201)

Mutant	CSH52	Su3	SuB	SuC	Probing	Mapping	Designation
M115	W	B	B	B	WT	P5	-G(957)
M116	B	B	B	B	WT	P1/P2	G(56) => A
M117	W	B	B	B	WT	P6	-G(735)
M118	W	B	B	B	WT	P5	-G(735)
M119	B	B	B	B	WT	P1/P2	-G(201)
M120	W	B	B	B	WT	P5	-G(588)
M122	W	B	B	B	WT	P3	-G413)
M123	W	B	B	B	WT	P5	-G(677)
M126	W	B	B	B	WT	P6	-G(735)
M128	B	B	B	B	WT	P1/P2	G(140) => T
M129	b	B	B	B	WT	P1/P2	-C(113)
M130	B	B	B	B	WT	P1/P2	-G(143)
M131	W	B	B	B	WT	P6	-G(783)
M132	W	B	B	B	WT	P6	-G(919)
M133	B	B	B	B	WT	P1/P2	-G(143)
M134	W	B	B	B	WT	P6	-C(988)
M135	W	B	B	B	WT	P3	-G(359)
M136	W	B	B	B	WT	P4	-C(493)
M138	B	B	B	B	WT	P1/P2	-C(186)
M139	B	B	B	B	WT	P1/P2	-G(143)
M140	W	B	B	B	WT	P6	-G(811)
M141	W	B	B	B	WT	P1/P2	-C(245)
M142	W	B	B	B	WT	P3	NC
M143	W	B	B	B	WT	P3	NR
M144	W	B	B	B	WT	P1/P2	-C(245)
M145	W	B	B	B	WT	PL	NC
M146	W	B	B	B	WT	P4	-G(422)
M147	B	B	B	B	WT	P1/P2	-G(143)
M149	W	B	B	B	WT	P6	-G(919)
M150	W	B	B	B	WT	P3	-G(437)
M151	W	B	B	B	WT	P6	G(842) => A
M152	b	B	B	B	WT	P6	NR
M153	W	B	B	B	WT	P6	-G(919)
M155	B	B	B	B	WT	P1/P2	-G(201)
M156	W	B	B	B	MINUS	P5	NR
M158	B	B	B	B	WT	P1/P2	G(93) => A
M160	B	B	B	B	WT	P1/P2	-C(186)
M161	W	B	B	B	WT	P6	-G(919)

I.4) CM6114 Mutations

Mutant	CS552	Su3	SuB	SuC	Probing	Mapping	Designation
N1	B	B	B	B	WT	P1/P2	NR
N2	W	B	B	B	WT	P4	-G(413)
N3	W	B	B	B	WT	P4	NC
N4	W	W	W	W	WT	P7	C(959) => T
N5	W	B	B	B	WT	P5	-G(677)
N6	W	B	B	B	WT	P5	NC
N7	B	B	B	B	WT	P1/P2	NR
N8	W	B	B	B	WT	P4	-C(591)
N9	W	B	B	B	WT	P1/P2	-C(245)
N10	B	B	B	B	WT	P1/P2	-C(202)
N11	W	B	B	B	WT	P1/P2	Deletion (267-282)
N12	W	B	B	B	WT	P4	C(528) => A
N13	W	B	B	B	WT	P6	-C(879)
N14	W	B	W	W	WT	P3	G(341) => T
N15	W	B	B	B	WT	P1/P2	Deletion (267-282)
N16	B	B	B	B	WT	P6	NR
N17	B	B	B	B	WT	P1/P2	-C(92)
N18	W	B	B	B	WT	P6	-G(919)
N19	W	B	B	B	WT	P1/P2	-G(300)
N20	B	B	B	B	WT	P1/P2	G(200) => T
N21	W	B	B	B	WT	P6	-G(811)
N22	W	B	B	B	WT	P4	-C(493)
N23	B	B	B	B	WT	P1/P2	Deletion (187-189)
N24	B	B	B	B	WT	P1/P2	-C(92)
N25	W	B	B	B	WT	P6	-C(745)
N26	W	b	b	b	WT	P1/P2	C(258) => A
N27	W	B	B	B	WT	P4	-G(588)
N28	W	B	B	B	WT	P6	-G(919)
N29	B	B	B	B	WT	P1/P2	-C(92)
N30	W	B	B	B	WT	P7	-G(972)
N31	B	B	B	B	WT	P1/P2	A(81G82) => TT
N32	W	B	B	B	WT	P1/P2	Deletion (267-282)
N33	B	B	B	B	WT	P1/P2	NR
N34	W	B	B	B	WT	P1/P2	Deletion (267-282)
N35	W	B	B	B	WT	P4	NR
N36	W	B	B	B	WT	P6	-G(919)
N37	W	B	B	B	WT	P6	-G(783)
N38	B	B	B	B	WT	P3	G(381) => A
N39	W	B	W	W	WT	P5	G(677) => T
N40	b	B	B	B	WT	P1/P2	G(222) => T
N41	W	B	B	B	WT	P1/P2	-G(300)
N42	W	B	B	B	WT	P1/P2	-C(245)
N43	B	B	B	B	WT	P1/P2	-T(172)
N44	B	B	B	B	WT	P1/P2	T(195) => A
N45	B	B	B	B	WT	P1/P2	-C(92)
N46	W	B	B	B	WT	P7	Deletion (929-943)
N47	W	W	B	B	WT	P3	C(318) => A
N48	W	B	B	B	WT	P3	NR
N49	B	B	B	B	WT	P1/P2	-C(186)
N50	W	B	B	B	WT	P5	NR
N51	W	B	B	B	WT	P6	C(795) => A

Mutant	CSH52	Su3	SuB	SuC	Probing	Mapping	Designation
N52	W	B	B	B	WT	P6	-G(758)
N53	B	B	B	B	WT	P1/P2	-C(92)
N54	W	B	B	B	WT	P6	C(750) => A
N55	B	b	B	b	WT	P1/P2	G(143) => T
N56	W	B	B	B	WT	P3	-G(391)
N57	W	B	B	B	WT	P6	-C(879)
N59	W	B	B	B	WT	P4	-C(591)
N60	b	B	B	B	WT	P1/P2	-C(109)
N61	W	B	B	B	WT	P6	-G(758)
N62	W	B	B	B	WT	P6	-C(879)
N63	B	B	B	B	WT	P1/P2	-C(92)
N64	W	B	B	B	WT	P4	-C(493)
N65	W	B	B	B	WT	P7	-G(919)
N66	W	B	B	B	WT	P7	NC
N67	W	B	B	B	WT	P6	-C(745)
N69	w	B	B	B	WT	P6	C(750) => A
N70	B	B	B	B	WT	P1/P2	C(104) => A
N71	W	W	B	B	WT	P6	T(939) => A
N72	W	B	B	B	WT	P1/P2	-G(391) (T(367) => A)
N73	w	B	B	B	WT	P6	Deletion (929-943)
N74	b	B	B	B	WT	P1/P2	G(221) => T
N75	b	B	B	B	WT	P1/P2	-G(134)
N76	W	B	B	B	WT	P6	-C(879)
N78	W	B	B	B	WT	P6	-C(745)
N80	B	B	B	B	WT	P1/P2	C(75) => A
N81	B	B	B	B	WT	P1/P2	C(80) => A
N82	B	B	B	B	WT	P1/P2	NC
N83	W	B	B	B	WT	P1/P2	-C(235)
N84	B	B	B	B	WT	P1/P2	C(174) => G
N85	B	B	B	B	WT	P1/P2	-G(201)
N86	B	B	B	B	WT	P1/P2	-C(92)
N87	b	B	B	B	WT	P1/P2	NC
N88	W	B	B	B	WT	P1/P2	-C(235)
N89	w	B	B	B	WT	P1/P2	-C(113)
N90	W	B	B	B	WT	P1/P2	-C(235)
N91	b	B	B	B	WT	P1/P2	-G(134)
N92	W	B	B	B	WT	P4	-C(591)
N93	W	B	B	B	WT	P6	-G(735)
N94	B	B	B	B	WT	P1/P2	C(270) => A
N95	B	B	B	B	WT	P1/P2	-C(67)
N96	W	B	B	B	WT	P7	-G(972)
N97	W	b	B	b	WT	P1/P2	C(258) => A
N98	W	B	B	B	WT	P6	-C(879)
N99	W	B	B	B	WT	P4	-C(496)
N100	W	B	B	B	WT	P4	-C(591)
N101	W	B	B	B	WT	P7	-G(972)
N102	B	B	B	B	WT	P1/P2	G(185) => T
N103	W	B	B	B	WT	P4	C(528) => A
N104	B	B	B	B	WT	P1/P2	A(203) => T
N105	B	B	B	B	WT	P1/P2	-C(175)
N106	b	B	B	B	WT	P1/P2	-G(134)
N107	W	B	B	B	WT	P6	-C(745)

Mutant	CSH52	Su3	SuB	SuC	Probing	Mapping	Designation
N108	W	B	B	B	WT	P4	G(537) => A
N109	B	B	B	B	WT	P1/P2	-C(113)
N110	B	B	B	B	WT	P1/P2	C(92) => A
N111	W	B	B	B	WT	P1/P2	-C(245)
N112	B	B	B	B	WT	P1/P2	C(186) => A
N113	W	B	B	B	WT	P4	Deletion (339-357)
N115	B	B	B	B	WT	P1/P2	C(57) => A
N116	W	B	B	B	WT	P6	-G(919)
N117	B	B	B	B	WT	P1/P2	G(221) => T
N118	W	B	B	B	WT	P6	NC
N119	W	B	B	B	WT	P6	-C(745)
N120	W	B	B	B	WT	P4	NR
N121	W	B	B	B	WT	P1/P2	-C(235)
N122	B	B	B	B	WT	P1/P2	G(116) => T
N123	B	B	B	B	WT	P1/P2	C(186) => A
N124	W	B	B	B	WT	P4	NR
N125	W	B	B	B	WT	P4	-C(493)
N126	W	B	B	B	WT	P6	-C(745)
N127	B	W	W	W	WT	P1/P2	C(210) => A
N128	W	B	B	B	WT	P1/P2	-C(252)G(253)
N129	W	B	B	B	WT	P3	-C(745)
N130	W	B	B	B	WT	P6	-A(898)
N131	W	B	B	B	WT	P6	-G(972)
N132	B	B	B	B	WT	P1/P2	A(110) => T
N133	W	B	W	W	WT	P3	G(326) => T
N134	B	B	B	B	WT	P1/P2	T(89) => A
N135	W	B	B	B	WT	P3	-C(493)
N136	W	B	B	B	WT	P1/P2	+G(134)
N137	B	B	B	B	WT	P1/P2	-C(175)
N138	W	B	B	B	WT	P4	-G(577C578)
N139	W	B	B	B	WT	P7	-G(919)
N140	B	B	B	B	WT	P1/P2	-C(175)
N141	W	W	W	W	WT	P6	G(731) => T
N142	W	B	B	B	WT	P6	-G(957)
N143	W	B	W	W	WT	P3	G(341) => T
N144	W	B	B	B	WT	P6	-C(892)
N145	W	B	B	B	WT	P6	-G(985)
N146	W	B	B	B	WT	P4	-C(493)
N147	W	B	W	W	WT	P6	C(867) => A
N148	w	B	B	B	WT	P6	C(693) => A
N149	W	B	B	B	WT	P5	-G(681)
N150	W	B	B	B	WT	P6	Deletion (828-837)
N151	W	B	B	B	WT	P4	-C(515)
N152	B	B	B	B	WT	P1/P2	G(93) => T
N153	B	B	B	B	WT	P1/P2	A(102) => T
N154	W	B	B	B	WT	P6	G(783) => T
N155	W	B	B	B	WT	P7	Deletion (980-1022)
N160	B	B	B	B	WT	P1/P2	C(120) => A
N161	B	b	W	W	WT	P1/P2	C(210) => A
N162	W	B	B	B	MINUS	P3	NR
N163	W	B	W	W	WT	P3	G(341) => T
N164	W	B	B	B	WT	P5	C(795) => A

Mutant	CSH52	Su3	SuB	SuC	Probing	Mapping	Designation
N165	W	B	B	B	WT	P5	Duplication (863-883)
N166	W	b	w	w	WT	P1/P2	C(318) => A
N167	W	B	B	B	WT	P3	-C(304)
N168	B	B	B	B	WT	P3	NC
N169	B	b	b	b	WT	P1/P2	G(143) => T
N170	B	B	B	B	WT	P1/P2	-C(92)
N171	W	B	B	B	WT	P5	-G(588)
N172	W	B	B	B	WT	P5	-G(671)
N173	B	B	B	B	WT	P1/P2	C(80) => A
N174	W	B	B	B	WT	P6	-C(745)
N175	W	W	W	W	WT	P1/P2	G(341) => T
N176	B	B	B	B	WT	P1/P2	G(221) => T
N177	B	B	B	B	WT	P1/P2	NC
N178	W	B	B	B	WT	P1/P2	-G(391)
N179	B	B	B	B	WT	P1/P2	C(174) => A
N180	W	B	B	B	WT	P7	-G(919)
N181	W	W	W	W	WT	P7	NR
N182	W	B	B	B	WT	P6	A(896) => T
N183	W	B	B	B	WT	PL	-G(919)
N184	B	B	B	B	WT	PL	NR
N185	W	B	B	B	WT	P7	-C(889)
N186	B	B	B	B	WT	P1/P2	C(90) => T
N187	B	B	B	B	WT	P1/P2	-G(143)
N188	W	w	W	w	WT	P1/P2	C(318) => A
N189	B	B	B	B	WT	P1/P2	C(75) => A
N190	W	B	B	B	WT	P7	G(843) => T
N191	W	B	B	B	WT	P6	-G(783)
N192	W	B	B	B	WT	P1/P2	-C(252G253)
N193	W	B	B	B	WT	P5	G(843) => T
N194	W	W	w	w	WT	P7	C(867) => A
N195	B	B	B	B	WT	P6	NR
N196	W	B	B	B	WT	P5	-C(879)
N197	B	B	B	B	WT	P1/P2	G(86) => C
N198	W	B	B	B	WT	P1/P2	-G(422)
N200	W	B	B	B	WT	P1/P2	-G(422)
N201	W	B	B	B	WT	P6	-C(879)
N202	B	B	b	b	WT	P1/P2	G(143) => T
N203	W	B	B	B	WT	P6	NR
N204	b	B	B	B	WT	P1/P2	-C(113)
N205	B	B	B	B	WT	P1/P2	-C(92)
N206	w	B	B	B	WT	PL	-C(1012)
N207	W	W	W	W	WT	PL	C(993) => A
N209	W	W	W	W	WT	P4	G(518) => T
N210	W	B	B	B	WT	P6	-G(783)
N211	W	B	B	B	WT	P7	-G(919)
N212	B	B	B	B	WT	P1/P2	C(92) => A
N213	B	B	B	B	WT	P1/P2	-C(188)
N214	b	W	W	W	WT	P1/P2	C(169) => A
N215	B	B	b	b	WT	P1/P2	G(134) => T
N216	b	B	B	B	WT	P1/P2	C(80) => A
N217	B	w	w	w	WT	P1/P2	C(210) => A
N218	W	B	B	B	WT	P6	-G(843)

Mutant	CSH52	Su3	SuB	SuC	Probing	Mapping	Designation
N219	B	B	B	B	WT	P1/P2	C(120) => A
N220	W	B	B	B	WT	P6	-G(985)
N221	B	B	B	B	WT	P1/P2	C(66) => A
N222	W	B	B	B	WT	P6	-G(735)
N223	W	B	B	B	WT	P6	-C(745)
N224	W	B	B	B	WT	P1/P2	-C(304)
N225	B	B	B	B	WT	P1/P2	C(174) => A
N226	B	B	B	B	WT	P1/P2	G(201) => T
N227	B	B	B	B	WT	P1/P2	G(201) => T
N228	W	B	B	B	WT	P6	C(750) => A
N229	W	b	w	b	WT	P6	C(867) => A
N230	W	B	B	B	WT	P7	-G(919)
N231	W	B	B	B	WT	P6	Deletion (917-969)
N232	W	B	B	B	WT	P6	-G(783)
N233	B	W	W	W	WT	P1/P2	G(59) => T
N234	B	B	B	B	WT	P1/P2	C(57) => A
N235	W	B	B	B	WT	P5	-C(879)
N236	W	B	B	B	WT	P4	-C(493)
N237	W	B	B	B	WT	P6	-G(783)
N238	W	B	B	B	WT	P7	-G(919)
N239	B	b	w	W	WT	P1/P2	C(210) => A
N240	W	B	B	B	WT	P6	-C(879)
N241	W	B	B	B	WT	P6	-C(745)
N242	W	B	B	B	WT	P6	-G(919)
N243	W	B	B	B	WT	P4	-C(493)
N244	W	W	W	W	WT	P3	C(318) => A

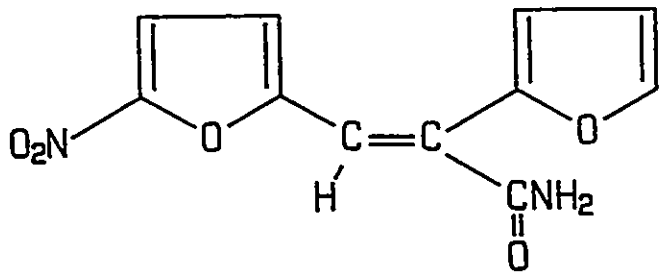
Appendix 2

THE MUTATIONAL SPECIFICITY OF AF2 IN THE LACI GENE OF E.COLI

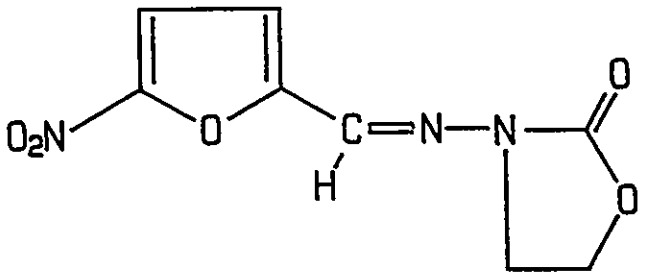
II.1 INTRODUCTION

Following World War II 5-nitrofuran derivatives were extensively used as antibacterial and antiprotozoal agents in human and veterinary medicine, and as food preservatives and additives (Bryan, 1978). In recent years, widespread use of 5-nitrofurans has been curtailed due to increasing evidence that several of these compounds are both mutagenic and carcinogenic (Tazima et al., 1975; Cohen, 1978; McCalla, 1983). One of the most potent nitrofuran derivatives in terms of mutagenicity is 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide (AF2) (Figure 36). This compound was used extensively in Japan as a human food preservative from 1966 until it was banned in 1974 following demonstration of its carcinogenicity in several rodent species (reviewed in Cohen, 1978, Tazima, 1979).

Reduction of the nitro moiety of 5-nitrofurans appears to be required for biological activity (McCalla, 1983). In both bacteria (Asnis, 1957; McCalla et al., 1970, Abraham et al., 1984) and mammalian cells (Wang et al., 1974, Swaminathan and Lower, 1978; Kutcher and McCalla, 1984; Mattamal et al., 1985) reduction is facilitated by two types of nitroreductases: those which are oxygen-insensitive and catalyze transfer of two electrons to the nitro moiety; and those which catalyze the transfer of 1 electron and are are inhibited by oxygen. Aminofurans and open chain nitriles have



2-(2-Furyl)-3-(5-nitro-2-furyl)-acrylamide (AF2)



Furazolidone

Figure 36. Structures of 5-nitrofuran derivatives.

been isolated as nongenotoxic endproducts of nitroreduction (Swaminathan and Lower, 1978, McCalla, 1983). Transient nitroso, hydroxylamino and open chain acrylonitrile derivatives are believed to be intermediate in the formation of these products (Abraham et al., 1984; Vroomen et al., 1988). Neither the DNA reactive species nor the resultant DNA adducts have been identified despite concerted efforts on the part of several laboratories. Recently, studies with furazolidone (Figure 36) have shown that the principal mercaptoethanol and glutathione conjugates are derived from reaction of the acrylonitrile intermediate (Vroomen et al., 1988). When administered to S. typhimurium tester strain TA100 the mercaptoethanol conjugate was found to be mutagenic, suggesting that the same reactive species might also form DNA adducts (Vroomen et al., 1988).

In bacterial mutagenicity tests, 5-nitrofurans are active base substitution mutagens. Nitrofurans induce reversion of E. coli trpA$^-$ (McCalla and Voutsinos, 1974) and S. typhimurium hisG46 strains (McCann et al., 1975). Mutation induction in E. coli is dependent on the induction of cellular error-prone repair (SOS) functions (Bryant and McCalla, 1980), while reversion of S. typhimurium tester strains is increased markedly in strains containing plasmid pKM101 (McCann et al., 1975; Green et al., 1977). The nitrofuran-induced mutation frequency is low in uvr$^+$ bacteria relative to otherwise isogenic strains which are deficient in nucleotide excision repair capability (Yahagi et al., 1974; McCalla and Voutsinos, 1974; Lu et al., 1979). The observations that the mutagenic potency of nitrofurans is

increased by the activity of SOS functions, and diminished in strains which are capable of carrying out nucleotide excision repair suggests that 5-nitrofurans form DNA adducts which: 1) are recognized and repaired by the uvrABC exinuclease; 2) act as an impediment to progression of the replication fork; and 3) can be bypassed in the presence of functions which diminish the fidelity of replication.

Studies of the specificity of forward mutation have been important in delineating potential mechanisms of mutation (Miller, 1983), and can provide information regarding the possible nature of the premutagenic lesions (Schaaper et al., 1987). In order to obtain a greater understanding of nitrofuran mutagenesis we have determined the precise DNA sequence changes induced by AF2 in the initial 180 base-pairs of the lacI gene of E. coli. The system used in these experiments is sensitive to several classes of mutation including base substitution, frameshift, deletion, insertion, and duplication (Burns et al., 1988a; 1988b; Schaaper, 1988).

II.2 MATERIALS AND METHODS

Strains and Media. E. coli strains NR3835: (F'lacpro; Δ(prolac) ara thi trpE9777); NR3951: Δ(bio uvrB) derivative of NR3835; and TC3960: plasmid pKM101 derivative of NR3951 were used for mutagenic treatment. The F'lacpro in these strains carry the I^Q (lacI) (Müller-Hill et al.,1968) and L8 (lacZ) (Scaife and Beckwith, 1966) promotor mutations. TC3960 was constructed in our laboratory by conjugal transfer of the plasmid pKM101 from S. typhimurium TA98 to NR3951 as described by Walker (1977). Media and strains used for cloning and mapping $lacI^-$ mutants have been previously described

(Chapter 3; Miller, 1972; Coulondre and Miller, 1977a; Schaaper et al., 1985).

AF2 Treatment. The treatment and selection procedures have been previously described (Burns et al., 1986; Burns et al., 1987). Multiple overnight cultures of the appropriate E. coli strain were started from single colonies. The following morning the cultures were diluted into fresh LB medium and grown to mid-log phase at which point the bacteria were pelleted by centrifugation and resuspended in Vogel-Bonner salt solution. AF2 (Abbott Laboratories), dissolved in DMSO, was added to a final concentration of 0.05-25 μM, and the cultures were incubated for 20 minutes at 37° C. Following treatment the cells were pelleted, washed, and appropriate dilutions of resuspended cultures were immediately spread on LB plates to determine survival, and on minimal plates supplemented with phenyl-β-D-galactopyranoside (PGal) (Research Organics Inc.) to select lacI$^-$ mutants. PGal is a non-inducing substrate for β-galactosidase: when supplied as the sole carbon source, only cells which constitutively express β-galactosidase (lacI$^-$ and lacO^c mutants) will form colonies. Plates were incubated at 37° C for 16 hours (LB plates) or 3 days (PGal plates). The short period of exposure to AF2 in buffer, followed by direct plating onto selective medium ensures the independence of the AF2-induced mutants. Mutant colonies were isolated from the PGal plates and mutations occurring within the initial 180 base-pairs of the lacI gene were identified by a simple dominance test (Chapter 3; Miller, 1972); these mutants were selected for DNA sequence analysis.

Cloning and Sequencing. The lacI$^-$ mutations were cloned by in vivo recombination from the F'lacpro episomal factor onto a specially constructed M13 vector mRS81 (lacI$^+$lacZ$_\alpha^-$) as described by Schaaper et al. (1985). Recombinant (lacI$^-$lacZ$_\alpha^+$) progeny were selected as blue plaques on an indicator E.coli strain plated on medium containing 5-bromo-4-chloro-3-indoyl-β-D-pyranogalactose (XGal) (Research Organics Inc.). Following purification and amplification of the recombinant phage, the single-stranded DNA of the mature virus was prepared by extraction with phenol/choroform/isoamyl alcohol (25:24:1). DNA sequence was determined by the dideoxy chain termination method (Sanger et al., 1980) using appropriate synthetic oligonuclectides (14-mers) as primers (Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton).

Other Methods. The stability of DNA secondary structures was examined using the computer program devised by Zuker and Steigler (1981). The thermodynamic parameters used for the prediction of duplex stability have been described recently by Freier et al. (1986).

II.3 RESULTS

The response (survival, and mutation to lacI$^-$) of NR3951, TC3960, and NR3835 to increased doses of AF2 was determined as described in Methods and Materials. The LD_{37} of AF2 was 0.65, 0.75 and 9.0 μM in NR3951 (Δuvr B), TC3960 (Δuvr B, pKM101) and NR3835 (wild type) respectively. The induced lacI$^-$ frequency at the LD_{37} in these experiments was 7 X 10^{-6} in the excision repair proficient strain

NR3835, 50 X 10^{-6} in NR2951, and 2300 X 10^{-6} in TC3960.

LacI$^-$ mutants were selected from TC3960 following AF2 treatment at a concentration of 1 μM. The average mutation frequency in the selection experiments was 1900 X 10^{-6} at 20 % survival. This represents an increase of 310-fold over the spontaneous mutation frequency (6 X 10^{-6}).

Although the lacI gene contains more than 1000 base pairs, mutations selected for DNA sequencing were limited to the initial 180 base pairs of the gene which encode the DNA binding domain of the lactose repressor. Mutations within this region (lacI^{-d}) are dominant to lacI$^+$ and can therefore be identified by a simple genetic test (Miller, 1972). The N-terminal region of the lactose repressor is extremely sensitive to base substitution mutations: DNA sequencing of several thousand lacI$^-$ mutations (Schaaper and Dunn, 1987; Schaaper, 1988; Gordon et al., 1988a; Glickman et al., unpublished results), in addition to previous studies of nonsense suppression (Miller, 1978), have shown that at least 140 different base substitutions yield the lacI$^-$d phenotype including 17 A:T => G:C, 22 A:T => T:A, 21 A:T => C:G, 23 G:C => A:T, 23 G:C => C:G, and 34 G:C => T:A sites. This represents over 50 % of all base substitution sites that have been found in the entire lacI gene. The DNA binding domain is also extremely sensitive to deletions, duplications, and frameshift mutations (Burns et al., 1988a; 1988; Schaaper, 1988).

LacI^{-d} mutations comprised 44 % of the AF2-induced mutants selected from TC3960. Taking into account the observation that less

than 15% of spontaneously arising *lacI*$^-$ mutations are dominant (Schaaper *et al.*, 1986; Halliday and Glickman, in preparation), it follows that AF2 increases *lacI*$^{-d}$ mutations by about 900-fold (310 X 44/15) over the spontaneous frequency.

A total of 165 *lacI*$^{-d}$ mutants were characterized by DNA sequence analysis (Table 28). Eighty-eight percent of the *lacI*$^{-d}$ mutations recovered following AF2 treatment were base substitutions. A detailed description of the base substitutions is given in Tables 29 and 30. Transversions outnumbered transitions by about 2:1. Of the 145 base substitution mutations characterized, 92 % occurred at G:C base pairs. These included 76 G:C => T:A transversions, 49 G:C => A:T transitions, and 10 G:C => C:G transversions.

More than one type of base substitution was recovered at eleven G:C sites (positions 56, 57, 75, 80, 84, 92, 93, 174, 178, 185, and 186). Mutations at these 11 sites accounted for 94 of the 135 base substitutions which occurred at G:C sites. The other 41 base substitutions involving G:C base pairs were recovered at 17 different sites. At 11 of these (positions 53, 65, 66, 134, 143, 147, 150, 169, 188, 191, and 206) the type of base substitution which was recovered is the only type of substitution known to yield a phenotype capable of growth on PGal medium. Six of the G:C sites where only a single type of base substitution was recovered (positions 116, 120, 140, 197, 198, and 201) are known to produce a selectable phenotype by more than one base substitution pathway.

A small number of base substitutions were also recovered at A:T base pairs. These included 9 A:T => T:A transversions, and 1 A:T

Table 28. Distribution of AF2-induced mutations by class

Mutation	Occurrences
Base Substitutions	145
Transversions	
G:C => T:A	76
G:C => C:G	10
T:A => A:T	9
Transitions	
G:C => A:T	49
T:A => C:G	1
Frameshifts	11
Complex	5
Tandem Base Subst.	2
Deletions	2
Total	165

Values are the number of independent occurrences of each type of mutation.

Table 29. Transversions induced by AF2 within the first 180 base pairs of the lacI gene.

Base Pair Change	Site[a]	Occurrences	Amino acid Change		Sequence[b]
G:C => T:A	53	1	GTC (Val)	TTC (Phe)	CGAT G ATGT
	57	18	GCA (Ala)	GAA (Glu)	CTCT G CGAC
	66	3	GCC (Ala)	GAC (Asp)	ACCG G CATA
	75	2	TCT (Ser)	TAT (Tyr)	ATAA G AGAC
	80	3	CAG (Gln)	AAG (Lys)	GTCT G ATAA
	84	6	ACC (Thr)	AAC (Asn)	AACG G TCTG
	92	3	CGC (Arg)	AGC (Ser)	ACGC G GGAA
	93	3	CGC (Arg)	CTC (Leu)	TCCC G CGTG
	116	3	GTT (Val)	TTT (Phe)	CCAC G TTTC
	134	2	GAA (Glu)	TAA (Ochre)	GCGG G AAAA
	143	1	GAA (Glu)	TAA (Ochre)	AGTG G AAGC
	147	1	GCG (Ala)	GAG (Glu)	CGCC G CTTC
	150	4	GCG (Ala)	GAG (Glu)	CATC G CGGC
	169	9	TAC (Tyr)	TAA (O8)[c]	GAAT G TAAT
	174	1	CCC (Pro)	CAC (His)	GTTG G GAAT
	178	1	AAC (Asn)	AAA (Lys)	CGCG G TTGG
	186	12	GCA (Ala)	GAA (Glu)	TTGT G CCAC
	198	2	GCG (Ala)	GAG (Glu)	GCCC G CCAG
	201	1	GGC (Gly)	GTC (Val)	GCGG G CAAA
Subtotal		76			
G:C => C:G	56	2	GCA (Ala)	CCA (Pro)	TGTC G CAGA
	65	3	GCC (Ala)	CCC (Pro)	GTAT G CCGG
	92	1	CGC (Arg)	GGC (Gly)	ACGC G GGAA
	178	2	AAC (Asn)	AAG (Lys)	CGCG G TTGG
	185	1	GCA (Ala)	CCA (Pro)	CGTG G CACA
	197	1	GCG (Ala)	CCG (Pro)	ACTG G CGGG
Subtotal		10			
A:T => T:A	54	2	GTC (Val)	GAT (Asp)	TGCG A CATC
	64	2	TAT (Tyr)	TAA (O4)	CGGC A TACT
	96	2	GTG (Val)	GAG (Glu)	CACC A CGCG
	141	1	GTG (Val)	GAG (Glu)	TTCC A CTTT
	167	1	TAC (Tyr)	AAC (Asn)	ATGT A ATTC
	183	1	GTG (Val)	GAG (Glu)	TGCC A CGCG
Subtotal		9			
Total		95			

[a] Numbering is according to Farabaugh (1978).
[b] The sequence given is the wild-type sequence of the purine containing strand and is read 5' => 3'.
[c] Nonsense mutation designation is that of Miller et al. (1978).

Table 30. Transitions induced by AF2 in the first 180 base pairs of the lacI gene.

Base Pair Change	Site[a]	Occurrences	Amino acid Change		Sequence[b]
G:C => A:T	56	1	GCA (Ala)	ACA (Thr)	TGTC G CAGA
	57	16	GCA (Ala)	GTA (Val)	CTCT G CGAC
	75	2	TCT (Ser)	TTT (Phe)	ATAA G AGAC
	80	1	CAG (Gln)	TAG (A5)[c]	GTCT G ATAA
	84	1	ACC (Thr)	ATC (Ile)	AACG G TCTG
	92	1	CGC (Arg)	TGC (Cys)	ACGC G GGAA
	93	1	CGC (Arg)	CAC (His)	TCCC G CGTG
	120	4	TCT (Ser)	TTT (Phe)	CGCA G AAAC
	140	2	GTG (Val)	ATG (Met)	AAAA G TGGA
	174	1	CCC (Pro)	CTC (Leu)	GTTG G GAAT
	185	2	GCA (Ala)	ACA (Thr)	CGTG G CACA
	186	13	GCA (Ala)	GTA (Val)	TTGT G CCAC
	188	2[d]	CAA (Gln)	TAA (O9)	TGTT G TGCC
	191	1	CAA (Gln)	TAA (O10)	AGTT G TGTG
	206	1	CAG (Gln)	TAG (A9)	GACT G TTTG
Subtotal		49			
A:T => G:C	195	1	CTG (Leu)	CCG (Pro)	CGCC A GTTG
Subtotal		1			
Total		50			

[a] Numbering is according to Farabaugh (1978).
[b] The sequence given is that of the purine containing strand and is read 5' => 3'.
[c] Nonsense mutation designation is that of Miller et al. (1978).
[d] Includes one mutation at which a second G:C => A:T transition occurred at position 100. The secondary mutation is at the third position of a valine codon and would be phenotypically silent. Since it is possible that this mutation was preexistent it will not be considered further.

=> G:C transition.

The distribution of base substitution mutations induced by AF2 is nonrandom. Table 31 shows that AF2-induced G:C => T:A transversions and G:C => A:T transitions appear to occur preferentially at guanine sites which contain a 5' or 3' pyrimidine. G:C => C:G transversions are also prefered at N-G-Y sites, but no effect of the 5'-flanking base was evident.

A large number of mutations were recovered at 5'-TGC-3' sites. Only 5 G:C base pairs in the initial 180 base pairs of the lacI gene contain guanine in this particular context. At two of these sites (positions 57 and 186), both G:C => T:A transversions and G:C => A:T transitions can be detected. A total of 59 base substitutions were recovered at these 2 sites. Three mutations were recovered at the one 5'-TGC-3' site (position 65) where only G:C => C:G transversions can be detected. An additional potential 5'-TGC-3' site exists at position 122, but since no mutations have been recovered at position 122 in this or any other study, it is likely that mutations at this position are phenotypically silent. Base substitutions at the last site (position 202) do not yield a phenotypically selectable lacI$^-$ mutation since position 202 is at the third position of a glycine codon. However, this site was found to be a frameshift hotspot (see below).

Several other classes of mutation were recovered following AF2 treatment. A detailed description of these mutations is given in Table 32. Single base frameshifts occurred at 2 different G:C sites. In neither case was the deleted base pair part of a reiterated

Table 31. The influence of flanking bases on the mutability of G:C base pairs.

		Mutations/detectable site[b]		
	Context[a]	G:C => T:A	G:C => A:T	G:C => C:G
5'-base				
	R-G-N	0.94	0.94	0.5
	Y-G-N	3.7	2.6	0.4
3'-base				
	N-G-R	0.86	1.1	0.16
	N-G-Y	3.2	2.7	0.52

[a] The context is taken from the strand containing guanine (underlined): R = purine; Y = pyrimidine; N = any base.

[b] detectable sites: the number of sites with a particular context which result in a selectable phenotype. These include sites where no mutations were detected in the present study. The data used in these calculation are as follows.

G:C => T:A transversion: 18 R-G-N, 16 Y-G-N, 14 N-G-R, and 20 N-G-Y sites.

G:C => A:T transition: 8 R-G-N, 15 Y-G-N, 8 N-G-R, and 15 N-G-Y sites.

G:C => G:C transversion: 8 R-G-N, 15 Y-G-N, 6 N-G-R, and N-G-Y sites.

Table 32. Summary of single base frameshift, tandem base substitution, complex, and deletion mutations recovered in the initial 180 base pairs of the lacI gene following AF2 treatment.

Mutation	Change	Site[a]	Sequence[b]	Occurrences
a) frameshifts	-G	147	TCGCC G CTTCC	1
	-G	202	TGTTT G CCCGC	10
b) tandem base subst.	CC => TT	173,174	ACATT CC CAACC	1
	TG => AT	99,100	CGTGG TG AACCA	1
c) deletions	-ACA	187-189	TGGC ACA ACAAC	2

d) Complex mutations

Mutation	Site[a]	Wild type sequence[b]	Mutant sequence
AF-89	127-129	TGCGAA AAC GCGGGA	TGCGAA TA GCGGGA
AF-113	104-106	GTGAAC CAG GCCAGC	GTGAAC AA GCCAGC
AF-143	135-140	CGGG AAAAAG TGGA	CGGG AAAAAAAAT TGGA
AF-158	123,128	TCTG C GAAA A CGCG	TCTG T GAAA C CGCG
AF-159	104,113	AAC C AGGCCAGC C ACG	AAC T AGGCCAGC T ACG

[a] Numbering is according to Farabaugh (1978).
[b] For frameshifts the sequence given is that of the purine containing strand; for tandem base substitutions, deletions, and complex mutations the sequence given is that of the nontranscribed strand and is read 5' => 3'.

sequence. One of these sites (position 202) was represented 10 times in the spectrum. The context of the deleted G (5'-TGC-3'on the transcribed strand) at this frameshift hotspot is identical to that of the base substitution hotspots described above.

Two tandem base substitution mutations were recovered (Table 32). In one case (at positions 173,174) the alteration was due to a double transition; while the other tandem base substitution (at positions 99,100) arose as the result of a double transversion.

A single deletion site was detected. The mutation in this case was the loss of the sequence 5'-ACA-3' (or 5'-CAA-3') from the sequence 5'-CACAACAAC-3'. This mutation was recovered twice following AF2 treatment.

Five mutations, all at different sites, resulted from multiple, closely spaced mutational events. Two of these, AF-158 and AF-159, each contained different base substitution mutations separated by 4 (AF-158) and 8 (AF-159) bases. The other three mutations were due to a combination of frameshift and base substitution events.

In one case (mutant AF-122) a secondary silent mutation (G:C => A:T at position 100) was characterized in addition to the primary base change (G:C => A:T at position 188). The secondary mutation is at the third position of a valine codon and would be phenotypically silent. Since the base substitution at position 188 can account for the i^{-d} phenotype by itself, the secondary mutation will not be considered further.

II.4 DISCUSSION

The ability of AF2 to induce mutation is highly dependent on cellular error-prone repair functions, and is quite low in strains which are proficient in nucleotide excision repair capability. In this study, the addition of the R-factor plasmid pKM101 to an excision repair deficient E. coli strain resulted in a marked increase (50-fold at the LD_{37}) in induced mutation frequency relative to bacteria lacking pKM101. The plasmid pKM101 contains the mucBA genes which are homologous to constituents of the endogenous umuCD locus, and are believed to encode functions which act during error-prone replication to facilitate progression of the replication fork past sites of DNA damage (Bridges and Woodgate, 1985). The observation that pKM101 enhances AF2 mutagenesis is consistent with previous studies in our laboratory which demonstrated that induction of the SOS response was required for reversion of trpA$^-$ E. coli (Bryant and McCalla, 1980). Similarly, in S. typhimurium, the AF2-induced mutation frequency is much higher in strain TA100 (hisG46, uvr$^-$, pKM101) than TA1535 (hisG46, uvrB$^-$) (McCann et al., 1975; Green et al., 1977). AF2-induced lethality and mutagenesis was dramatically reduced in strains with functional nucleotide excision repair (Results; McCalla and Voutsinos, 1974; Yahagi et al., 1974; Lu et al., 1979). This suggests that AF2 forms a DNA adduct which can be recognized and repaired by the uvrABC eximuclease of E. coli.

DNA sequencing of AF2-induced lacI^{-d} mutants provides us with insight regarding both the nature of the premutational adduct formed by AF2 and the consequences of mutagenic bypass of this lesion. One

hundred and fifty-six of the 165 mutants characterized in this study were the result of a single base change (145 base substituions and 11 frameshift mutations). Ninety-four percent of these point mutations occurred at G:C sites, suggesting that AF2 forms adducts preferentially with either guanine or cytosine. The most reactive base for several other mutagens which form bulky adducts (for example BPDE (Meehan et al., 1977), AF (Beland et al., 1983), AAF (Miller, 1978; Beland and Kadlubar, 1985), AFB_1-2,3-dichloride (Swenson et al., 1975), and 1,8-NONP (Chapter 2)) is guanine. It has previously been shown that mutagenic bypass of either bulky DNA adducts formed with guanine (Miller, 1983), or aguaninic sites (Kunkel, 1984) occurs with the preferential incorporation of adenine opposite the lesion, resulting in G:C => T:A transversions. The most common class of mutation induced by AF2 treatment was indeed G:C => T:A transversions. Thus the specificity of mutation is consistent with adduct formation occurring on a guanine residue.

Loeb (1985) has suggested that the apurinic (AP) site might be a common intermediate in mutagenesis induced by several agents that form bulky DNA adducts. During replication, progression of the DNA polymerase might be hindered by a DNA adduct until depurination occurs, with the resultant AP site presenting less of a barrier to continued (error-prone) replication. In the model proposed by Loeb the poised replication complex would protect the AP site from recognition and repair by AP endonuclease. Kunkel (1984) has demonstrated that the preference for base incorporation opposite AP sites is A > T > G > C. The mutational specificity of AF2 suggests

that mutation induced by this chemical might also proceed through an AP site. If all the base substitution mutations induced by AF2 (76 G:C => T:A transversions, 49 G:C => A:T transitions, and 10 G:C => C:G transversions) arose from bypass of damage on guanine residues then the ratio of incorporation opposite the lesion is A (56 %) : T (36 %) : G (7.5 %). This is remarkably similar to the specificity of base incorporation opposite aguaninic sites (A (56 %) : T (29 %) : G (15 %)) observed by Kunkel (1984) in the α-complementation segment of phage M13mp2. It may also be noted that 11 sites were represented by more than one type of base substitution, suggesting that different mutations at a particular site are induced by a common DNA adduct. If that adduct depurinated, then errror-prone replication past the resultant aguaninic site would be likely to yield different types of base substitution mutations. This interpretation would also be consistent with previous observations that AF2 and other mutagenic 5-nitrofuran derivatives induced alkali labile sites in DNA (McCalla et al., 1971; Tu and McCalla, 1975), and that alkali caused a marked reduction of supercoiled DNA in minicells treated with AF2 (Tu and McCalla, 1975).

If AP sites were also induced by a minor DNA adduct formed with adenine then it would be expected that an incorporation preference of A > T > G > C would yield a large proportion of A:T => T:A transversions, since insertion of T would not result in a mutation when inserted opposite an AP site derived from adenine. As shown in Tables 29 and 30, nine out of ten base substitutions at A:T sites were A:T => T:A transversions with 1 A:T => G:C transition.

Thus, the preference of base incorporation opposite adenine (A(90%) : G(10%) : C(0%)) compares favorably with the specificty of base incorporation opposite aadeninic sites determined by Kunkel (1984) (A(81%) : G(12%) : C(6%)).

Guanine residues within the sequence 5'-TGC-3' sites are hotspots for AF2 mutagenesis. Forty-three percent of all base substitution mutations were recovered at the three 5'-TGC-3' sites where base substitutions yield a selectable phenotype. Almost all of the frameshift mutations (10/11) occurred at position 202, a 5'-TGC-3' site at which base substitution mutations will not produce a mutant capable of growth on PGal. The frameshift mutation at position 202 is at a lone G:C base pair. This is different from many frameshift mutation hotspots for other agents, which contain reiterated DNA sequences. Several runs of contiguous guanine residues are present in the initial 180 base pairs of the lacI gene and are readily mutated by other chemicals which induce bulky adduct damage, such as BPDE (Bernelot-Moens, Glickman, and Gordon, submitted) and 1,8-NONP (Chapter 3), and also by uv irradiation (Miller, 1985; Schaaper et al., 1987), and intercalating agents such as ICR-191 (Calos and Miller, 1981). Thus it is unlikely that the frameshifts occurred by "typical" misalignment mechanisms (see Chapter 3 for a more complete discussion of frameshift mechanisms). In addition, since the vast majority of frameshifts were recovered at a single position (202), it is likely that it is the nature of this site that is important rather than the general propensity of AF2 to induce frameshift mutations. One interesting feature of this

frameshift mutation is that it can be explained by a novel DNA secondary structure. Figure 37 shows a large palindrome in which a 13 base sequence from positions 82-94 pairs almost perfectly (12/13 bases with the 1 mismatch being an energetically favourable G:T pair) with the mutant sequence. The free energy of the proposed structure (-43.6 kcal/mole) is favourable. Stalled replication at an AF2 lesion or an AF2-induced AP site at position 202 might lead to extended single-stranded regions which could fold to form intrastrand duplexes. DNA repair within this structure could result in mutation. For instance AP endonuclease might promote incision of the DNA adjacent to the AP site; subsequent excision, repair synthesis and ligation within the palindrome using the inverted repeat at positions 82-94 as a template would produce the observed mutation.

The precise nature of the DNA adduct(s) formed by AF2 or other 5-nitrofurans is not known. Although it has been possible to detect binding of [^{14}C]-labelled AF2 to DNA (Wentzell and McCalla, 1980), the instability of the adduct has hindered attempts to further characterize the lesion(s). As noted earlier, 5-nitrofurans are activated to DNA reactive species by reduction of the nitro moiety. An important intermediate during nitroreduction is the hydroxylamine. Studies with both nitroarenes and arylamines have shown that hydroxylamine derivatives of aromatic compounds can react with DNA leading to stable C(8) adducts of guanine (Chapter 2; Beland et al., 1983; Beland and Kadlubar, 1985; Kadlubar and Beland, 1985; Andrews et al., 1986). The mutagenic consequences of adduction at guanine C(8) are primarily frameshifts and G:C => T:A transversions (Chapter

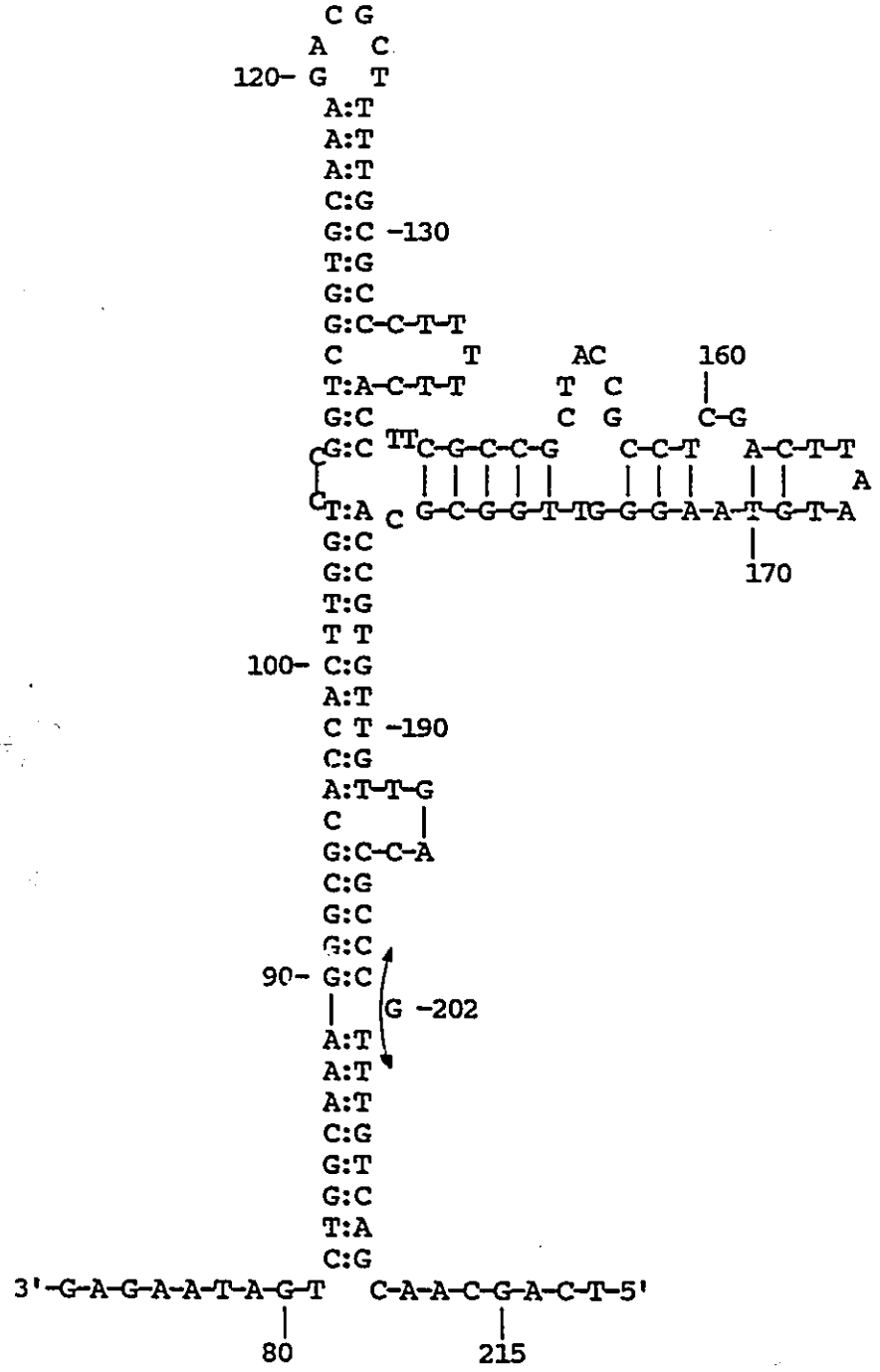


Figure 37. DNA secondary structure which predicts the frameshift mutation at position 202.
The strand shown is the transcribed strand. The numbering is according to Farabaugh (1978). The free energy for this structure is -43.6 kcal/mol.

3; Miller, 1983; Rosenkranz and Mermelstein, 1983; Koffel-Schwartz et al., 1984, Bichara and Fuchs, 1985). For nitrofurans an important alternative consequence of hydroxylamine formation is ring opening which ultimately leads to the formation of open chain nitrile derivatives (Figure 38). A reactive intermediate in this pathway is the acrylonitrile species which contains a reactive carbon. The acrylonitrile derivative of furazolidone has been shown to bind to biological macromolecules, and to induce mutations in S. typhimurium (Vroomen et al., 1988). An intriguing possibility is that the mutagenicity of 5-nitrofurans might be mediated by the binding of the reactive carbon of the acrylonitrile metabolite to the N(7) position of purines (Figure 38). Adducts at the N(7) position of purines are known to increase the lability of the N-glycosylic bond resulting in frequent depurination (Loeb and Preston, 1986). In this context it might be noted that the base substitution mutation spectrum observed with AF2 is quite similar to that of AFB_1-2,3-dichloride in M13AB28 lacZ DNA (Sambamurti et al., 1988), and BPDE in the lacI gene (Gordon, Bernelot-Moens, and Glickman, in preparation), and quite distinct from that of 1,8-NONP in the lacI gene (Chapter 3), or AF in the Tet^R gene of pBR322 (Bichara and Fuchs, 1985). Both AFB_1-2,3-dichloride and BPDE bind to the N(7) position of guanine (Swenson et al., 1975; Osborne et al., 1978); while 1,8-NONP and AF bind to the C(8) position of guanine (Chapter 2; Kriek, 1965; Andrews et al., 1986; Beland et al., 1983).

Depurination could also occur as a consequence of glycosylases acting on modified purine residues. It is known that

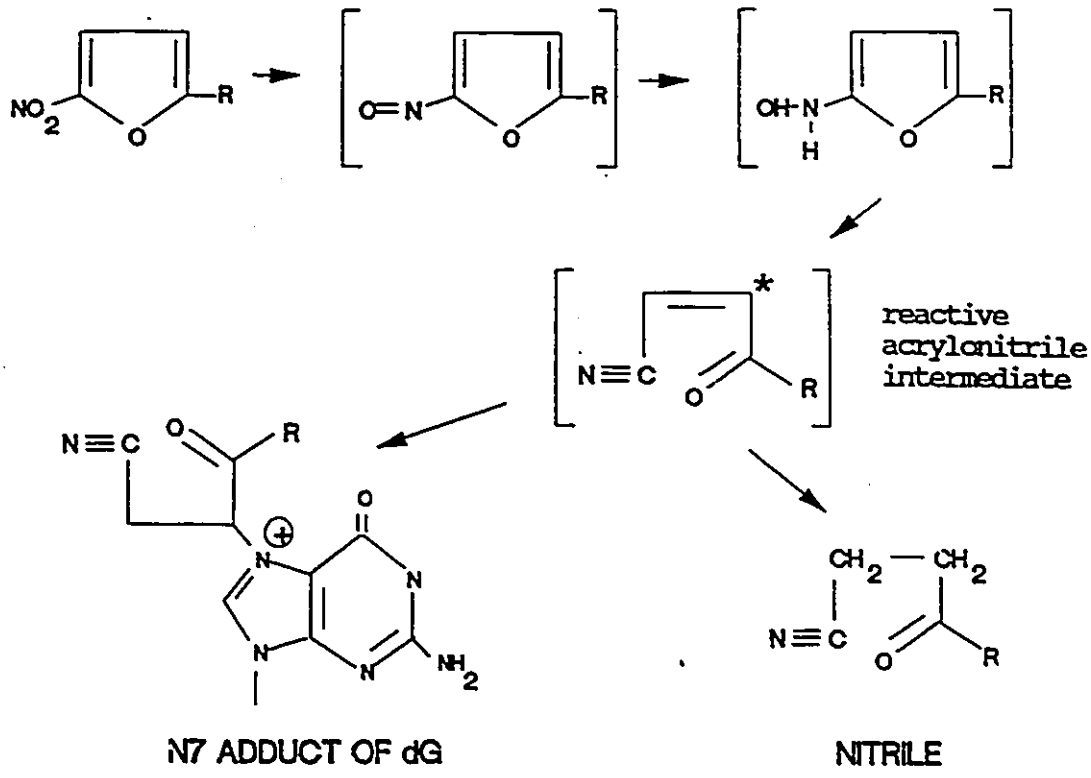


Figure 38. Hypothetical pathway for adduct formation by 5-nitrofurans.

imidazole ring-opened products of N(7)-modified (Chetsanga and Lindahl, 1979; Chetsanga et al., 1982; Chetsanga and Frennette 1983) and C(8)-modified (Boiteux et al., 1989) purine residues are substrates for E. coli formimidopyrimidine (Fapy)-DNA glycosylase. The product of this enzymatic reaction is an AP site. However, since most of the AP sites produced in this manner would be formed away from the replication fork, it is likely that the majority would be correctly repaired by AP endonuclease prior to replication.

AF2 mutagenesis has a strong site specificity. Mutations are particularly frequent at guanine sites which are flanked on either side by a pyrimidine. The site specificity could be attributable to preferential binding of AF2 to sites which are flanked by pyrimidines. Other mutagens are known to bind preferentially to guanines in particular sequences. For instance, BPDE binding to the N(7) position of guanine occurs preferentially at sites preceded by a pyrimidine (Lobanenkov et al., 1986) while adduction at the N(7) position of guanine by AFB_1-2,3-dichloride is strongly preferred in runs of guanine (Muench et al., 1983). Alternatively the site specificity might be ascribed to differential depurination of adducts within particular sequences.

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