INVESTIGATION OF THE MECHANISM OF MUTAGENESIS

BY THE DNA ADDUCTS OF THE

HUMAN BLADDER CARCINOGEN, 4-AMINOBIPHENYL

by

SUSAN BINA MALAIKAL VERGHIS

B.S., Tufts University, 1982

SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY
FEBRUARY 1992

Signature of Author _____
Division of Toxicology
January 28, 1992

Certified by _____
John M. Essigmann
Thesis Advisor

Accepted by _____
Steven R. Tannenbaum
Chairperson, Committee on Graduate Students

This doctoral thesis has been examined by a Committee of the Division of Toxicology as follows:

Professor Steven Tannenbaum _____ Chairperson

Professor John Essigmann _____ Thesis Supervisor

Professor Helmut Zarbl _____

Professor Peter Lansbury _____

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ABSTRACT

This study investigated the mutagenicity of the major DNA adduct formed by the human bladder carcinogen 4-aminobiphenyl (ABP), *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8-ABP), in the bacteriophage M13mp19 single-stranded (ss) genome replicated in *Escherichia coli*. To determine if DNA adducts formed by ABP could induce mutations in the M13 ss genome, two complementary mutational analyses in *E. coli* were undertaken. In the first study, mutagenesis assays in the *lacZ* gene of globally ABP-modified M13mp10 ss and duplex (ds or RF) DNAs were conducted to determine which of the known DNA adducts of ABP were likely to be mutagenic in the M13mp10 ss genome and to compare the ABP mutational spectrum in ss DNA to that established in the *lacZ* gene of ABP-modified RF DNA. M13 globally ABP-modified ss and RF DNAs were replicated in excision-repair proficient (*uvr*$^+$) and excision-repair deficient (*uvr*$^-$) *E. coli* strains that were induced or uninduced for SOS mutagenic processing functions. The results showed ABP-DNA adducts in ss DNA were approximately 70-fold more mutagenic and 50-fold more genotoxic than ABP lesions in ds DNA in SOS-induced *uvr*$^+$ cells. ABP-DNA adducts in M13 ss DNA were dependent on the *mucAB* processing genes for enhanced mutagenesis in SOS-induced cells. An analysis of the ABP-induced mutations indicated that only base-pair substitutions were detected in the *lacZ* gene of ABP-modified ss DNA. Over 80% of the mutations occurred at putative dG-ABP adducts. Among the base changes, G -> T transversions were the predominant mutations followed by G -> C transversions and then G -> A transitions.

In the second study, site-specific mutagenesis assays were conducted with an M13 ss genome containing a single dG-C8-ABP lesion to determine the genotoxic and mutagenic effects of the major ABP-DNA adduct. A

deoxytetranucleotide, 5'-d[pTpG-(ABP)pCpA]-3', containing a single dG-C8-ABP adduct was synthesized, characterized and incorporated into an M13mp19 gapped duplex genome at a unique *Pst*I site. The adduct was situated in either the plus strand at position 6245, or in the minus strand at position 6246 of the vector. The TG-(ABP)CA tetramer ligated into the genome with an efficiency of 25%, compared to a 50% ligation efficiency for a control genome. After *in vivo* replication of both the ABP-modified and the control ss genomes in *E. coli* strains, DL7 (*uvr*$^+$) and DL7/pGW16 (*uvr*$^+$/*mucAB*), mutants were isolated to determine the mutation frequency and the mutation specificity of the dG-C8-ABP lesion. The results of three methods of mutant selection have indicated that the targeted mutation frequency of a single dG-C8-ABP lesion, situated at the *Pst*I site in an M13 ss genome, was approximately 0.01%. The primary mutation observed at this site was the G -> C transversion. This mutation occurred with equal frequency from putative adducts originating from either the plus strand or from the minus strand of the genome.

These studies concluded that although the dG-C8-ABP was weakly mutagenic under these experimental conditions, the contribution of this lesion to a portion of the mutational spectrum induced by ABP in ss DNA is plausible.

Thesis supervisor: Professor John M. Essigmann

Title: Professor of Toxicology and Professor of Chemistry

ACKNOWLEDGEMENTS

First and foremost, I would like to dedicate this thesis both to my parents, Annie and Matthew Malaikal and especially to my late parents-in law, Rachel and George Verghis. Their love, support and faith in me have helped me make all this possible.

I am ever grateful to my thesis advisor, Prof. John M. Essigmann for his guidance, support, academic intuition and friendship during my academic career at MIT. At times, his faith in my academic abilities was greater than my own. I would also like to thank members of my thesis committee, especially my chairman, Prof. Steve Tannenbaum for his critical comments.

I would also like to thank Dana Lasko for her advice and technical expertise, both of which have been helpful in my research project. I also want to thank Dr. Fred Kadlubar of the NCTR, who, in addition to being a gracious host, was extremely helpful to me during my visit to Arkansas in 1987.

In the Essigmann laboratory, I have many individuals to thank for their intellectual and technical assistance, including Ashis Basu, Lisa Naser Bradley, Brian Donahue, Dipti Mathur, Marshall Morningstar, Anil Purohit, Wendy Rowell, Daniel Treiber, Jennifer Wilson, and Michael Wood. Members of other laboratories that have also been helpful include, Nazneen Aziz, Cathy Costello, Ed Loechler, Pete Wishnok and Pat Zarrinkar. I want to thank Denise Harrington for her Mac-expertise in helping me prepare the figures and tables for my thesis. I also want to thank Kim Bond Schaefer and Laurie MacLeod and Debra Luchanin for their administrative support.

Finally, I want to especially thank my husband, Mammen George and my dear daughter, Rachel Nina for all their love, support and sacrifices they have made so that I may achieve my academic goals. Their friendship, good humor, and bright outlook on life has made completion of this dissertation much easier.

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LIST OF ABBREVIATIONS

AAAF	*N*-Acetoxy-*N*-acetyl-2-aminofluorene
AAF	*N*-Acetyl-2-aminofluorene
ABP	4-Aminobiphenyl
AF	2-Aminofluorene
AP	apurinic/apyrimidinic
b.c.	buffer control conditions
bp	base pair
BZ	Benzidine
CIP	calf intestinal alkaline phosphatase
dA	deoxyadenosine
dA-C8-ABP	*N*-(deoxyadenosin-8-yl-4-aminobiphenyl
dC	deoxycytosine
dG	deoxyguanosine
dG-C8-ABP	*N*-(deoxyguanosin-8-yl-4-aminobiphenyl
dG-C8-AAF	*N*-(deoxyguanosin-8-yl)-*N*-acetyl-2-aminofluorene
dG-C8-AF	*N*-(deoxyguanosin-8-yl-2-aminofluorene
dG-N^2-ABP	*N*-(deoxyguanosin-N^2-yl)-4-aminobiphenyl
dT	deoxythymine
ds	double-stranded
E. coli	*Escherichia coli*
EDTA	disodium salt of ethylenediaminetetraacetic acid
form I	supercoiled RF (ds) DNA
form II	nicked circular RF (ds) DNA
form III	linear RF (ds) DNA
Hb	Hemoglobin
IPTG	isopropyl-β-D-thiogalactopyranoside
LB	Luria-Bertani broth
2-NA	2-Naphthylamine
NCTR	National Center for Toxicological Research
NO-ABP	Nitrobiphenyl
N-OAc-ABP	*N*-Acetoxy-4-aminobiphenyl
N-OAc-TFAABP	*N*-Acetoxy-*N*-trifluoroacetyl-4-aminobiphenyl
N-OH-ABP	*N*-hydroxy-4-aminobiphenyl
PHS	Prostaglandin H synthase
RF	replicative form
ss	single-stranded
S. typhimurium	*Salmonella typhimurium*
TE	10 mM Tris-HCl (pH 8.0), 1 mM EDTA
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
uvr$^+$	excision repair-proficient
uvr$^-$	excision repair-deficient
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
wt	wild-type

I. INTRODUCTION

Most, if not all, chemical carcinogens undergo metabolic or spontaneous chemical activation to electrophilic derivatives that go on to form a variety of addition products, or adducts, with the DNA of cells (Miller and Miller, 1981). Most chemical adducts formed in DNA are removed by cellular repair enzymes prior to replication of the genetic material; however, defects in the repair or the removal of certain adducts occasionally result in mutations. Many studies over the past decade have suggested that these genetic changes activate cellular proto-oncogenes or inactivate tumor suppressor genes (Weinberg, 1989; Vogelstein, 1990). These initial mutagenic events are important because they may lead to neoplastic transformation of cells which ultimately may result in carcinogenesis.

One important goal of mutagenesis studies using chemical carcinogens is to evaluate the genotoxicity induced by a carcinogen *in vivo*. Results from this type of evaluation will help to elucidate the mechanism and genetic requirements for the mutational specificity of a carcinogen. More specifically, these studies can serve as models for understanding the cellular factors that cause cells to resist, or to succumb to, the genotoxic properties of a toxic compound.

The objective of my research, which is documented in this dissertation, was to investigate the mechanism of the repair and/or the mutagenesis of the DNA adducts of the human bladder carcinogen, 4-aminobiphenyl (ABP). The bacteriophage M13 was the model for study of ABP mutagenesis in two

complementary mutation analysis systems. In the first, forward mutation of the full range of ABP adducts was studied in *Escherichia coli* using the *lacZα* gene of M13 bacteriophage single-stranded (ss) DNA as the genetic target; mutants were isolated and the molecular nature of the mutations was determined. These data helped formulate hypotheses as to which DNA adducts were the likely precursors to ABP-induced mutagenesis. The second line of approach tested one of those hypotheses by introducing the major DNA adduct of ABP into a known site in an M13 ss genome, followed by replication of this uniquely-modified genome in *E. coli*. This approach permitted an assessment of the mutational specificity of the major ABP-DNA adduct as well as its contribution to the entire mutational spectrum induced by ABP in *E. coli*.

The studies presented in this dissertation follow a collaboration initially formed between our laboratory and Dr. Fred F. Kadlubar, renowned for his expertise in the chemistry and pharmacokinetics of aromatic amines, at the National Center for Toxicological Research (NCTR). Although this research project has not investigated all aspects of the mutagenesis and the repair of ABP-DNA adducts, it has led to a furthering of our understanding of the genotoxicity of a chemical that is increasingly being appreciated for its adverse impact on public health.

II. LITERATURE SURVEY

Aromatic Amine Compounds and Carcinogenesis

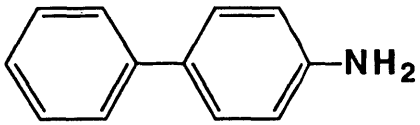
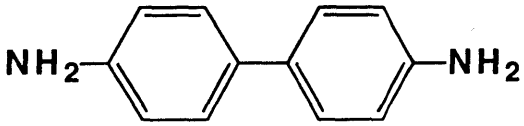
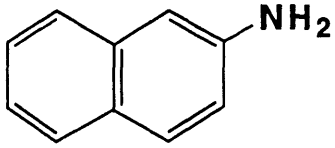
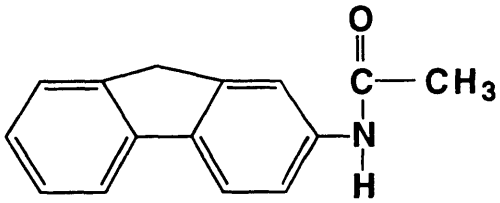
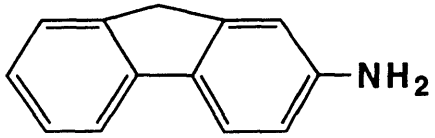
This literature survey will review DNA adduct formation and mutagenesis caused by an number of *N*-substituted aromatic compounds that have been widely studied in the field of chemical carcinogenesis. The metabolism of aromatic amine compounds and the role of these chemicals in carcinogenesis will also be reviewed. The survey will focus on the following aromatic amine compounds: 4-aminobiphenyl, benzidine (BZ), *N*-acetyl-2-aminoflourene (AAF), 2-aminofluorene (AF) and 2-naphthylamine (2-NA). The structures of these aromatic amine compounds and their applications in industry or their occurrence in the environment are shown in Figure 1.

History of Industrial Exposure to Aromatic Amines

The aromatic amines and related compounds are known to be potent carcinogens (IARC, 1972; 1987). Among these, 4-aminobiphenyl, has been shown to be a potent bladder carcinogen in humans (Melick et al., 1955; 1971). ABP was manufactured and used in this country as an antioxidant in the rubber manufacturing industry from 1935 until 1954 (Garner et al., 1984). Its production and use was ceased when it was implicated as the etiological agent in bladder cancer induced in industrially exposed workers (Melick et al., 1955). The results of the study performed by Melick and coworkers demonstrated that a striking 19 of 171 (11%) workers exposed to

Figure 1.

Structures of Aromatic Amine Compounds and their Industrial Use or Occurrence in the Environment (from King, 1982).

Aromatic amine compound	Use / Occurrence
 4-aminobiphenyl (ABP)	antioxidant; cigarette smoke
 benzidine (BZ)	dye intermediate
 2-naphthylamine (2-NA)	antioxidant; cigarette smoke
 N-Acetyl-2-aminofluorene (AAF)	insecticide
 2-aminofluorene (AF)	synthetic fuels

ABP in industrial use had developed bladder tumors. A follow-up study of 315 exposed workers revealed that approximately 17% of this larger group had developed bladder cancers (Melick et al., 1971). These data were compiled from individuals exposed at two plants owned by the only company that was known to produce and use ABP compounds in this country (Garner et al., 1984).

In addition to ABP, other aromatic amines, such as 2-naphthylamine (2-NA) have also been implicated in industrial cases of bladder cancer, which were originally believed to be due to exposure to aniline (Case et al., 1954). The aromatic amine, benzidine (BZ), was used in the production of dyes in the chemical industry for nearly 100 years. Although the incidence was lower, bladder tumors due to industrial exposure to BZ was also reported (Case et al., 1954). Benzidine is an intermediate in the production of several widely used carcinogenic dyes: Direct Black 38, Direct Blue 6 and Direct Brown 95 (Garner et al., 1984). A related aromatic compound, developed as a potential insecticide, that has been extensively studied but never used is *N*-acetyl-2-aminofluorene (AAF). AAF and cogeners were found to be carcinogenic in experimental animals prior to their synthesis in large-scale quantities that would have resulted in industrial, and then general population, exposure (Wilson et al., 1941).

Animal Models for Aromatic Amine Carcinogenesis

In addition to the role of aromatic amines in the development of human cancers, the carcinogenic potency of these compounds have been extensively investigated in experimental animals. The first report that provided

evidence for the carcinogenicity of aromatic amines in experimental animals was conducted by Hueper and coworkers (1938). The results of this study clearly demonstrated that dogs given 2-NA developed bladder cancer. Subsequently, many studies were conducted with animals to test the carcinogenicity as well as to elucidate the mechanisms of carcinogenesis by ABP and related aromatic amines. Prior to the implication of ABP in the etiology of industrial bladder cancer in this country, the carcinogenic potential of ABP was demonstrated in both experimental rats and dogs by Walpole and coworkers in Great Britain (1952; 1954). The results suggested that the dog was the most susceptible animal to develop bladder cancer compared to other animals such as rats or rabbits (Walpole et al., 1954; Block et al. 1978). Rabbits develop bladder cancers although at a lower incidence than in dogs (Radomski, 1979) and rats were observed to primarily form mammary and liver tumors when given ABP (Dooley et al., 1988).

The results of later studies with 2-NA indicated that, overall, this compound is most carcinogenic in the dog, followed by rabbits, guinea pigs and hamsters, with the rat being the least susceptible to 2-NA carcinogenesis (Radomski, 1979). BZ and its derivatives cause mammary tumors (Morton et al., 1981) and urothelial tumors in rats (Wang et al., 1990). The carcinogenic nature of AAF was initially demonstrated in rats through studies conducted by Wilson et al. (1941). Subsequently, AAF was found to be carcinogenic in dogs, in which it induces liver and bladder tumors; rats and hamsters develop a variety of tumors, including liver and bladder neoplasms, and rabbits form bladder and uterine tumors when treated with AAF or its metabolites (King, 1982).

The carcinogenic potency of ABP to induce bladder tumors in dogs is greater than those of 2-NA, BZ or AAF; in rats, however, AAF appears to be the most potent hepatocarcinogen followed by ABP, 2-NA and BZ (Radomski, 1979; Garner et al., 1984).

Based on the experimental and clinical evidence provided by many researchers, most carcinogenic aromatic amines have been removed from industrial use. This point not withstanding, the prevalence of bladder cancer in certain areas of the world has been well documented (Parkes and Evans, 1984). The aromatic amines continue to be in the environment, and thus exposure of humans to these compounds is an everyday occurrence for many individuals (Kadlubar et al., 1988c).

Environmental Exposure to Aromatic Amine Compounds

Aromatic Amines in Tobacco Smoke

As indicated above, ABP is no longer used in industry, but recent studies have shown that ABP may still pose a carcinogenic risk from its presence as an environmental contaminant. One known contemporary route of ABP exposure is through cigarette smoke (Bryant et al., 1987). Many carcinogens, including aromatic amines such as ABP and 2-NA (both of which are known bladder carcinogens) have been detected in cigarette smoke. ABP is present in the mainstream smoke produced from blond tobacco cigarettes at 2.4 ng per cigarette. In cigarettes containing black tobacco the amount of ABP is 4.6 ng in the mainstream smoke and 143 ng in the sidestream smoke (Patrianakos and Hoffman, 1979).

Cigarette smoking has long been associated with an increased risk for bladder cancer (Mommsen and Aagaard, 1983; Vineis et al., 1984; Vineis et al., 1988), lending further, albeit indirect, evidence for the significance of the aromatic amines, including ABP, as agents that might be responsible for the myriad of toxic effects of smoking. Among bladder cancer patients, smokers of black tobacco (air-cured) had a 2-3 fold increased risk of bladder cancer compared to blond (flue-cured) tobacco cigarette smokers (Vineis et al., 1988). These results correlate with the finding that black tobacco contains a higher concentration of carcinogens as compared to blond tobacco (Patrianakos and Hoffman, 1979).

Smoking-Related ABP-Hemoglobin Adducts

One measure of exposure to ABP from tobacco smoke is the analysis of ABP and related compounds in the bloodstream of cigarette smokers. The results of early studies by the Tannenbaum laboratory have determined that smokers, compared to nonsmokers, have an increased level of ABP residues in their blood as measured by ABP bound to hemoglobin (Hb) to form ABP-Hb adducts (Bryant et al., 1987). A mean value of 154 pg of ABP/per g of Hb in blood samples from smokers versus 28 pg ABP/g Hb for nonsmokers was determined in this study. A later report by the same group indicated that after withdrawl from cigarette smoking, the levels of the ABP-Hb adduct in smokers declined to the levels observed in nonsmokers (Maclure et al., 1990). This study clearly demonstrated that Hb adducts of ABP were formed from exposure to cigarette smoke. In studies comparing adduct levels among individuals that smoked different types of tobacco, it was determined that

the levels of ABP-hemoglobin adducts in smokers of black tobacco were higher than the ABP-Hb adducts formed from smokers of blond tobacco (Bryant et al., 1988). Again, this finding is consistent with the higher concentration of carcinogens in black tobacco cigarette smoke compared to blond tobacco smoke (Patrianakos and Hoffman, 1979), as well as to the increased risk for bladder cancer in smokers of black tobacco versus blond tobacco smokers (Vineis et al., 1988).

Recent studies have demonstrated that ABP can pass across the human placenta in pregnant women and bind to fetal hemoglobin to form ABP-Hb adducts (Coghlin et al., 1991). The results of this study definitively showed that levels of ABP-Hb adducts were higher in both the maternal blood samples as well as the fetal blood samples from smokers compared to nonsmokers. A important finding reported in many studies, was that ABP-Hb adducts were present in minor, but perhaps significant amounts in blood samples from nonsmokers as well (Bryant et al., 1987; Kadlubar, 1988b). Thus, it appears that ABP exposure continues today to a large number of people both directly and passively with exposure to cigarette smoke and perhaps other environmental sources. The measurement of ABP-Hb adducts in blood samples from individuals can be used as a biomarker as well as a dosimeter for exposure to ABP and other relevant environmental carcinogens.

Exposure to Related Aromatic Amine Compounds

Aside from tobacco smoke, exposure to aromatic amines and related compounds may also result from other sources. For example, the carcinogens, AF, ABP, and 2-NA have been reported to be found in coal-

derived synthetic fuels (Guerin and Buchanan 1987). The recent manufacture and use of benzidine-based dyes has also been documented (Schulte et al., 1988; Mirokova and Lalchev, 1990). The presence of the structurally related compounds, the heterocyclic amines, have been detected in cooked or fried high-protein foods such as meat and fish (Sugimura and Sato, 1983). Heterocyclic amines have been demonstrated to be activated to DNA binding compounds similarly to aromatic amines (Kato and Yamazoe, 1990; Turesky et al., 1991). These, in addition to as yet unknown sources of aromatic amines and related compounds, are believed to be important in the etiology of human cancer (Parkes and Evans, 1984; Weisburger, 1988; Kadlubar et al., 1988c).

Metabolism of Aromatic Amine Compounds

Susceptibility to Carcinogenesis by Aromatic Amines

Although many individuals are exposed to aromatic amines and related compounds on a frequent basis, there may be other factors such as genetic susceptibility and pharmacokinetics in the metabolism of these chemicals which may be important in the etiology of aromatic amine carcinogenesis. One of the goals of recent studies has been to establish a relationship between inter-individual metabolism of carcinogenic agents and subsequent tumor formation as a result of exposure to these agents in various individuals (Kadlubar et al., 1988c). Enzymatic capabilities in the detoxification or in the activation of carcinogenic compounds appear to play an important role in species or organ specificity of aromatic amine-induced carcinogenesis (Harris, 1989).

Aromatic amines induce tumors infrequently at sites of administration. Usually these compounds affect distal tissues, mainly liver, intestine, and bladder indicating that metabolic activation of aromatic amines is essential for their carcinogenicity (Miller, 1970). The use of experimental animals as models for the study of the metabolism of aromatic amine carcinogens in humans has led to a further understanding of the mechanisms of metabolic activation and metabolic detoxification of these compounds (Miller, 1978).

The Role of N-Acetylation Reactions in the Metabolism of Aromatic Amines

It is generally accepted that the carcinogenic basis of aromatic amines and related compounds depend on the metabolic activation of these compounds by the host organism (Miller and Miller, 1981). Various pathways including *N*-hydroxylation, *N*-acetylation and *N*-deacetylation are important in the activation of aromatic amines to tumorigenic agents (King and Glowinski, 1983; Gorrod and Manson, 1986). The capacity for *N*-acetylation by liver *N*-acetyltransferases involved in the metabolism of aromatic amines is hypothesized to be important in species and organ specificity of tumorigenesis by these compounds (King and Glowinski, 1983).

Studies attempting to establish the relationship between acetylation of aromatic amines, acetylator phenotype, and target organ tumorigenesis have suggested that acetylation leads to activation of carcinogens in species prone to arylamine-hepatocarcinogenesis whereas in species susceptible to arylamine-induced bladder cancer, acetylation represents a detoxification pathway (Hein, 1988; Kadlubar et al., 1988c; Flammang et al., 1989).

Interspecies differences have been observed in the metabolism and the carcinogenic effects of aromatic amines in various tissues (Radomski, 1979). In rats, which are known to possess *N*-acetylation enzymes, liver tumors are evident after treatment with ABP or its derivatives (Miller et al., 1961). In dogs, bladder tumors from aromatic amines are predominant, which may be due to the fact that these animals do not acetylate ABP metabolites and therefore are not susceptible to tumors in the liver, where *N*-acetylation reactions occur (Poupko et al., 1983). *In vitro* studies using dog liver microsomes demonstrated enzymatic deacetylation of *N*-acetylated-ABP compounds. This metabolic pathway may render dogs susceptible to liver and bladder tumors from acetylated aromatic amines such as AAF and bladder tumors from deacetylated aromatic compounds such as ABP (Lower and Bryan, 1976). The ability to *N*-acetylate aromatic amine bladder carcinogens is believed to be an important detoxification step in the metabolism of ABP; however, with compounds such as BZ, *N*-acetylation represents a activation step (Morton et al., 1979; Weber and Hein, 1985).

In addition to the enzymatic capacity for *N*-acetylation by liver *N*-acetyltransferses, genetic differences in the rates of *N*-acetylation of various aromatic amines have also been observed in rabbits (McQueen et al., 1982; Ilett et al., 1991), and mice (Mattano et al., 1988). Cell culture studies with rabbit hepatocytes indicate that rabbits that were shown to have high rates of acetylation, designated the fast acetylator phenotype, were more susceptible to the genotoxic effects of AF and BZ than were the slow acetylator phenotypes (McQueen et al., 1982; 1983). Recently, it has

been demonstrated that liver or colon cytosol preparations from rabbits with the rapid acetylator phenotype when incubated with either N-OH-ABP or N-OH-AAF and DNA resulted in significantly higher DNA binding than preparations from slow acetylator rabbits (Ilett et al., 1991). A higher carcinogen-DNA adduct level in certain tissues could suggest an increased susceptibility of arylamine carcinogenesis in these organs.

The aromatic amine *N*-acetyltransferase also shows a genetic polymorphism in humans; thus affecting the rates of *N*-acetylation of various aromatic amine carcinogens (Flammang et al., 1988; Land et al., 1989). Fast acetylator phenotypes are homozygous dominant or heterozygous for two alleles of the gene for the *N*-acetyltransferase; slow acetylators are homozygous recessive for the two alleles (Hein, 1988). Recently, the two alleles found in slow acetylators have been identified as M1 and M2. These are associated with a decrease in the levels of the *N*-acetyltransferase enzyme rather than the presence of a defective enzyme (Blum et al., 1991).

The ability to acetylate aromatic amines may or may not predispose humans to carcinogenesis from exposure to these compounds. An early study by Cartwright et al. (1982) demonstrated that among industrial workers exposed to aromatic amines, 22 of 23 individuals that developed bladder cancer were of the slow acetylator phenotype. In the same study, these investigators also reported that among individuals that were not occupationally exposed to aromatic amines, there was no association with bladder carcinomas and acetylator phenotype. The results of later studies have indicated a correlation between acetylator phenotype and bladder

cancer risk, with an increased number of bladder cancers observed in individuals with a slow acetylator phenotype (Mommsen et al., 1985; Mommsen and Aargard, 1986).

Other studies have investigated the relationship between acetylation phenotype and colorectal carcinoma. A higher proportion of rapid acetylator phenotypes among colorectal cancer patients compared to matched controls was reported in one study (Ilett et al., 1987). Recent studies, however, have shown very little correlation between the fast acetylator phenotype and the susceptibility to colorectal carcinoma (Kirlin et al., 1991).

Further studies in this area may unravel this complex issue; however, the basic conclusions from these studies indicate that individuals with the fast acetylator phenotype may be more susceptible to liver and colon carcinogenesis, whereas slow acetylator phenotypes appear to be prone to bladder carcinogenesis. Inter-individual differences observed in the metabolism of the carcinogens, ABP, AAF, AF or BZ may lead to differences in the nature of the tumorigenicity induced by these compounds.

Recent studies have investigated the relationship between cigarette smoking, acetylation phenotype and the formation of carcinogen adducts in human urothelial cells and blood samples. In the genetically determined slow acetylators, there was an increase in the levels of ABP-Hb adducts present in blood samples that correlated with the type of tobacco smoked by the individual (Vineis et al., 1990). The results of the study suggested that the slow acetylator phenotype could be at a higher risk for arylamine-induced bladder cancer due to the increased exposure to ABP as evidenced by

the higher number of ABP-Hb adducts formed in these individuals. Recent reports have documented a correlation between carcinogen-protein binding to carcinogen binding of cellular DNA (reviewed in Skipper and Tannenbaum, 1990). Inter-individual differences in the metabolic activation of carcinogenic compounds and the formation of carcinogen-DNA adducts have been reported (Harris, 1989).

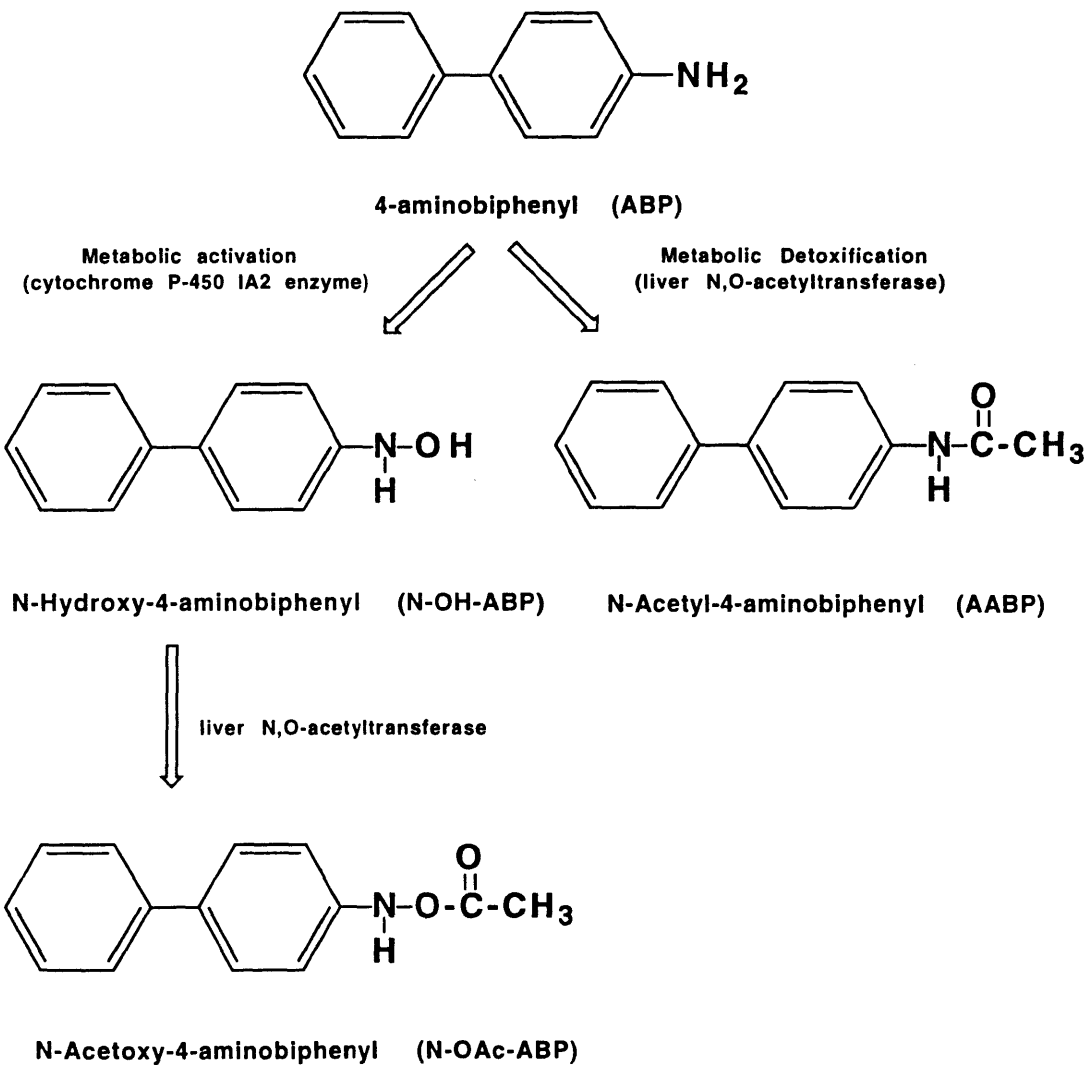
Metabolic Activation of Aromatic Amines by N-Hydroxylation

In addition to the importance of *N*-acetylation in the metabolism and tissue susceptibility to tumorigenesis by aromatic amines, in most species, metabolic activation of these compounds by *N*-oxidation by P-450 mixed-function oxidases is believed to be a critical step in the conversion of the promutagen to the proximate carcinogen (Cramer et al., 1960; Radomski et al., 1973; Miller and Miller, 1981).

The initial step of metabolic activation of aromatic amines such as ABP is through *N*-oxidation to a more hydrophilic species, *N*-hydroxy-4-aminobiphenyl (N-OH-ABP) (Figure 2) (Miller et al., 1961; Hammons et al., 1985; Guengerich and Shimada, 1991). The detoxification reactions of ABP that occur mainly via liver *N*-acetyltransferases compete with the formation of N-OH-ABP by liver monooxygenases. Detailed investigations have demonstrated that purified liver cytochrome P-450 isoenzymes are active in the oxidation of ABP to N-OH-ABP (Masson et al., 1983; Guengerich et al., 1988). Recently, in humans, N-hydroxylation of ABP was demonstrated to be catalyzed predominantly by the liver cytochrome P-450 IA2 enzyme (Butler et al., 1989a; 1989b).

Figure 2.

Proposed Pathways of Metabolic Activation of ABP by N-hydroxylation or Detoxification of ABP by N-acetylation in Humans (based upon Beland and Kadlubar, 1986).


NH2
4-aminobiphenyl (ABP)
Metabolic activation
(cytochrome P-450 IA2 enzyme)
Metabolic Detoxification
(liver N,O-acetyltransferase)
N-OH
H
O
N-C-CH3
H
N-Hydroxy-4-aminobiphenyl (N-OH-ABP)
N-Acetyl-4-aminobiphenyl (AABP)
liver N,O-acetyltransferase
O
N-O-C-CH3
H
N-Acetoxy-4-aminobiphenyl (N-OAc-ABP)

The metabolism of related aromatic amines has been studied in parallel with ABP. The carcinogen AAF is activated *in vivo* in two steps: *N*-hydroxylation of AAF by P-450 enzymes results in formation of N-OH-AAF, this is followed by esterification by *N*-acetyltransferases or sulfotransferases to form the highly reactive N-acetoxy-AF or the sulfuric acid ester, respectively (Kriek and Westra, 1979; Miller and Miller, 1981). Alternatively, N-OH-AAF could be deacetylated by liver enzymes to form the reactive N-hydroxy-AF compound (King and Glowinski, 1983).

In most species, BZ compounds are *N*-acetylated to *N*-acetylbenzidine (ABZ) by liver *N*-acetyltransferases before *N*-hydroxylation by P-450 enzymes occurs (Morton et al., 1979). ABZ and DABZ have been shown to be better substrates than BZ for the liver P-450 enzymes (Hammons et al., 1985).

Genetic variations in the ability to activate arylamines through *N*-oxidation by the cytochrome P-450 enzymes has been observed in humans (Butler et al., 1989a). A combination of the rapid arylamine oxidizer/slow acetylator phenotype has been suggested to be more susceptible to arylamine-urinary bladder carcinogenesis (Kadlubar et al., 1990). An individual with this phenotype may not be able to rapidly detoxify aromatic amines such as ABP. Consequently, the fast oxidation of these compounds would result in them being transported via the circulation to the bladder where they could form adducts with cellular DNA. A recent study demonstrated that a combination of a slow acetylator phenotype with a fast metabolic oxidizer phenotype formed the highest number of ABP-Hb adducts (Bartsch et al., 1990). Since *N*-oxidation is a critical initial step in the activation of arylamines which leads to the formation of Hb adducts,

the presence of these adducts could be used as a biomarker of exposure to aromatic amine carcinogens.

Metabolism of Aromatic Amines in In Vitro Cell Systems

The metabolic activation of aromatic amines has been studied in both animal models and cell culture systems. Studies using isolated liver microsomes from different species have demonstrated that *N*-hydroxylation by hepatic monooxygenases is an important step in the metabolic pathway for most animals (McMahon et al., 1980). Bladder epithelial (urothelial) cells from rats, cattle and dogs have been shown to have the capacity to *N*-hydroxylate aromatic amines (Poupko et al., 1983). The results of *in vitro* studies with isolated hepatocytes from different species have shown that the extent of *N*-hydroxylation of ABP correlates with the occurrence of N-hydroxylated-ABP compounds in the urine of the same species (Jatoe and Gorrod, 1987).

Other studies have shown that *N*-hydroxylated metabolites of ABP and AF were effective in inducing more unscheduled DNA synthesis in cultured dog urothelial cells compared to rats or rabbits (Wang et al., 1985; Wang et al., 1988a, 1988b). The results of these studies correlate with earlier investigations which indicate that dogs are more susceptible than rats or rabbits to arylamine-bladder carcinogenesis (Radomski, 1973).

The carcinogen-induced cytotoxicity of the *N*-hydroxylated metabolites of ABP has been demonstrated in experiments performed with cultured human uroepithelial cells (HUC) (Reznikoff et al., 1986). The findings confirm that *N*-hydroxylation of ABP is an essential step in the

activation of this compound to a carcinogenic agent. More recent experiments using HUC have shown that N-OH-ABP can be metabolized to ABP and 4-acetylaminobiphenyl (AABP) (Swaminathan and Reznikoff, 1988; Swaminathan, 1988). These results indicate that N-OH-ABP could be activated by urothelial *N*-acetyltransferases, indicating a role for the bladder in the metabolism of ABP and related derivatives.

The activation of ABP to reactive metabolites in target tissues has been investigated using purified bladder microsomal preparations (Poupko et al., 1983). The results indicate that species differences exist in the activation of ABP by bladder mucosa. Monooxygenases capable of activating ABP were not found in the dog bladder, suggesting that hepatic *N*-oxidation is a more relevant route for ABP metabolism in dogs.

In addition to *N*-oxidation in the liver as an activation step, recent evidence suggests metabolism of ABP can occur in the target organ, dog bladder, via the peroxidase-mediated pathway. *In vitro* studies have demonstrated peroxidative activation of ABP and other bladder carcinogens by prostaglandin H synthase (PHS), an enzyme present in dog urothelium (Kadlubar et al., 1982b). Peroxidase activation of ABP has been observed in studies with human bladder and dog bladder explants incubated with ABP (Kadlubar et al., 1988a). Activation of BZ by PHS has been demonstrated in dog urothelial cells (Wise et al., 1984) as well as in human bladder microsomal preparations (Flammang et al., 1989).

Metabolism of ABP in Dogs

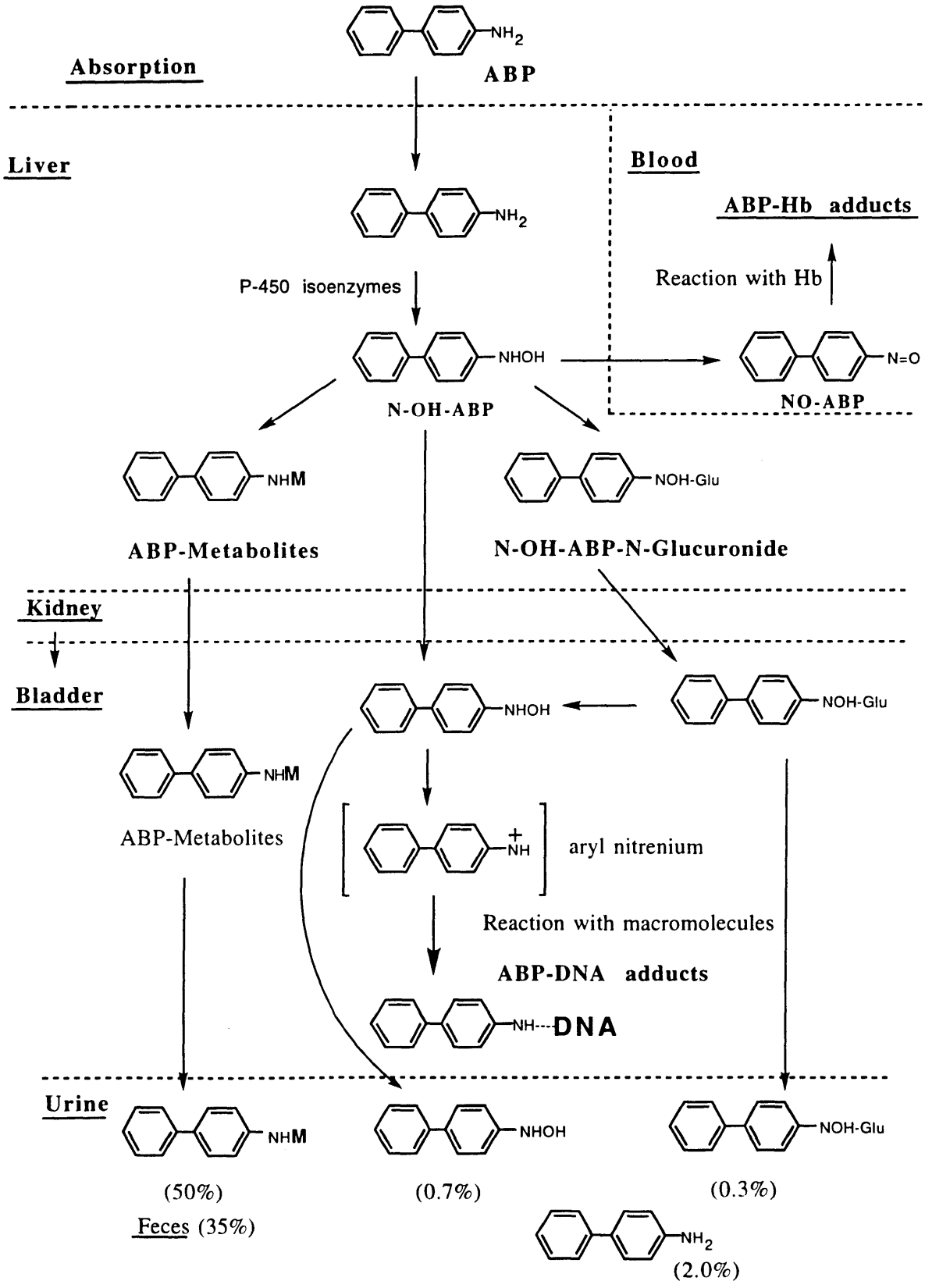
Activation and metabolism of ABP has been studied using dogs as a model because of similarities to humans in the pathogenesis of bladder carcinogenesis (Block et al., 1978; Beland et al., 1983). In dogs, after initial hepatic *N*-oxidation of ABP to N-OH-ABP, some of this species becomes conjugated to a glucuronide moiety in the liver, and either the resulting conjugate, *N*-hydroxy-ABP-*N*-glucuronide or the unconjugated N-OH-ABP is transported to the bladder for subsequent elimination (Radomski et al., 1977; Poupko et al., 1979; Kadlubar et al., 1977; Kadlubar et al., 1991).

Initial studies had suggested that the majority of the N-hydroxy-ABP is conjugated to the glucuronide; however, recent evidence indicates that the most of the *N*-OH-ABP is filtered into the bladder as the free hydroxylamine, while the glucuronide conjugate represents only 0.3% of the total ABP entering the bladder lumen (Figure 3) (Kadlubar et al., 1988b; Kadlubar et al., 1991). In dogs as well as in humans, it is believed that some fraction of the *N*-glucuronide moiety may break down in the bladder to form N-OH-ABP. The hydroxylamine compound is believed to undergo a change to form a reactive metabolite presumed to be an aryl-nitrenium ion, which binds to nucleic acids in bladder epithelial cells (Kadlubar et al., 1977; Kadlubar et al., 1991).

Recent evidence has shown that urine voiding interval is an important factor in the susceptibility to bladder tumorigenesis by ABP in dogs. Higher levels of urothelial DNA adducts of ABP were obtained from animals

Figure 3.

A Model for the Metabolism and Distribution of Metabolites of ABP and the Formation of ABP-DNA and ABP-Hemoglobin Adducts in Dogs (adapted from Kadlubar et al., 1991).


Absorption
ABP
Liver
Blood
ABP-Hb adducts
Reaction with Hb
P-450 isoenzymes
NHOH
N=O
N-OH-ABP
NO-ABP
NHM
NOH-Glu
ABP-Metabolites
N-OH-ABP-N-Glucuronide
Kidney
Bladder
NHOH
NOH-Glu
NHM
ABP-Metabolites
aryl nitrenium
Reaction with macromolecules
ABP-DNA adducts
NH---DNA
Urine
NHM
NHOH
NOH-Glu
(50%)
(0.7%)
(0.3%)
Feces (35%)
NH2
(2.0%)

that were subjected to longer intervals before urine voiding (Kadlubar et al., 1987, Kadlubar et al., 1988b; Kadlubar et al., 1991). These studies provide evidence that in the dog bladder, ABP-DNA adducts are formed from N-OH-ABP as a function of urine retention time more than as a function of urine pH.

The metabolism of other bladder carcinogens has also been studied in dogs. Since BZ is a bladder carcinogen, and dogs have been found to be unable to acetylate arylamines (Poirier et al., 1963; Lower and Bryan, 1976), other routes of BZ metabolism in dogs have been investigated. One predominant route of activation of BZ is through peroxidative metabolism in extrahepatic tissues by PHS (Kadlubar et al., 1982b; Wise et al., 1984). A recent study investigated the possible pathways of tumor induction by BZ in dogs (Lakshmi et al., 1990). The authors concluded that *in vivo* oxidation by PHS is a likely route of BZ metabolism in dogs (Lakshmi et al., 1990).

Formation of DNA Adducts of Aromatic Amine Compounds

Once chemical carcinogens are metabolized, these metabolites can react with different sites in cellular DNA to form a variety of carcinogen-DNA adducts (Miller and Miller, 1981). Chemical-DNA lesions that are not repaired by cellular enzymes may serve as precursors to mutations during replication of DNA (Loeb, 1989). Inter-individual variation in the metabolism of chemical carcinogens, in the spectrum of formation of chemical-DNA adducts of carcinogens, as well as in the repair of carcinogen-DNA lesions play a significant role in individual susceptibility

to cancer development (Harris, 1989; 1991). Knowledge of the individual's genetic predisposition to cancer as well as improved methods in the detection of carcinogen-DNA adducts may provide the best methods for risk assessment (Kadlubar et al., 1988c).

The study of DNA adduct formation by ABP and related carcinogens has been well studied both *in vitro* with cell culture systems and *in vivo* with experimental animals. The formation of DNA adducts of ABP may represent an initial step in the formation of tumors induced by ABP. One study has demonstrated that ABP can be transferred from the blood into the milk of lactating female rats; this may represent a significant source of exposure to the nursing offspring of these rats (La Voie et al., 1989). As might be expected, liver ABP-DNA adducts were detected in the nursing pups as a result of this ABP exposure. Another study using the technique of ^{32}P-Postlabeling of DNA demonstrated that ABP can traverse the placenta of pregnant mice and form DNA adducts in fetal organs (Lu et al., 1986).

The assay of carcinogen-DNA adducts as biomarkers for exposure has been demonstrated successfully using a noninvasive procedure to analyze the DNA originating from exfoliated urothelial cells (Talaska et al., 1990). A very recent study has detected the levels of ABP-DNA adducts to be 2-20 fold higher in the exfoliated urothelial cells from smokers versus nonsmokers (Talaska et al., 1991b). In addition, this report indicated a positive correlation with the presence of urothelial DNA adducts and the mutagenicity of urine in bacterial mutagenesis assays.

Spectrum of ABP-DNA Adduct Formation

The formation of DNA adducts of ABP has been extensively studied in dogs as the model system, because of similarities to man in the development of bladder tumors after exposure to ABP and other aromatic amines (Beland and Kadlubar, 1986).

There are three major DNA adducts formed *in vivo* when ABP is administered to dogs (Kadlubar et al., 1982a; Beland and Kadlubar, 1985). For ABP adduct analysis, the DNA from dog bladder was isolated, and both the extent of binding and the adduct profile were determined. The urothelial DNA was enzymatically hydrolyzed and the resultant nucleosides were analyzed by high pressure liquid chromatography (HPLC). The adducts were identified as *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8-ABP), *N*-(deoxyguanosin-N^2-yl)-4-aminobiphenyl (dG-N^2-ABP), and *N*-(deoxyadenosin-8-yl)-4-aminobiphenyl (dA-C8-ABP); these adducts represent approximately 75%, 15%, and 10% of the binding *in vivo* to DNA, respectively (Figure 4).

An analysis of both dog urothelial and liver DNA indicated that ABP-DNA adduct binding increased between 1 and 2 days after ABP administration and remained constant for another 5 days (Beland et al., 1983; Beland and Kadlubar, 1985) in both liver and bladder DNA. In dogs, only the bladder and not the liver has been reported to be a target for tumor formation by ABP (Radomski, 1979).

A similar distribution of binding *in vitro* was observed when *Salmonella typhimurium* was treated with (N-OH-ABP) or when N-OH-ABP was

Figure 4.

Structures of the DNA Adducts of 4-Aminobiphenyl (from Beland and Kadlubar, 1985).

reacted, at pH 5, with calf thymus DNA (Figure 4) (Kadlubar et al., 1982a; Beland et al., 1983; Beland and Kadlubar, 1985).

In addition to ABP and N-OH-ABP, other reactive ABP compounds have been used to form ABP-DNA adducts (Figure 5) (Beland et al., 1983; Lasko et al., 1988). When the reactive metabolite of ABP, *N*-acetoxy-*N*-trifluoroacetyl-ABP (N-OAc-TFAABP) was reacted with DNA, the major adduct was determined to be the dG-C8-ABP adduct (Lasko et al., 1988; Beland et al., 1990; Tamura and King, 1990).

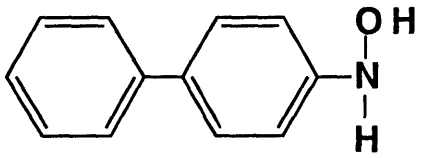
In an experiment performed with HUC cytosol fractions incubated with N-OH-ABP, acetyl-CoA and calf thymus DNA, the major ABP-DNA adduct formed was identified as the dG-C8-ABP adduct (Swaminathan and Reznikoff, 1988). The results of this study demonstrate the potential capacity of the human bladder in ABP metabolism and DNA adduct formation.

Recent studies have demonstrated that the predominant carcinogen-DNA adduct detected in biopsy tissue samples from urinary bladders of cigarette smokers was identical to a synthetic ABP-deoxyguanosine (dG) adduct, dG-C8-ABP, based on chromatographic data (Talaska et al., 1991a). In addition, the dG-C8-ABP adduct detected in exfoliated urothelial cells from dogs given ABP was chromatographically identical to that found in exfoliated urothelial cells from smokers (Talaska et al., 1990; Talaska et al., 1991b).

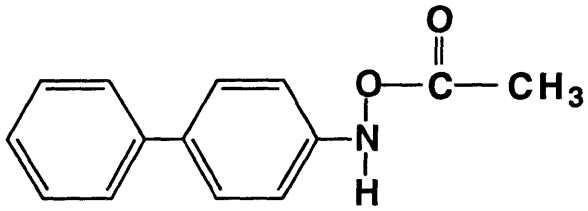
The crucial step in the activation of ABP and resulting DNA adduct formation is by *N*-oxidation catalyzed by the P-450 oxygenases; however, studies have demonstrated that purified PHS enzyme from dog urothelium was also able to activate ABP to form DNA adducts (Kadlubar et al., 1988a).

Figure 5.

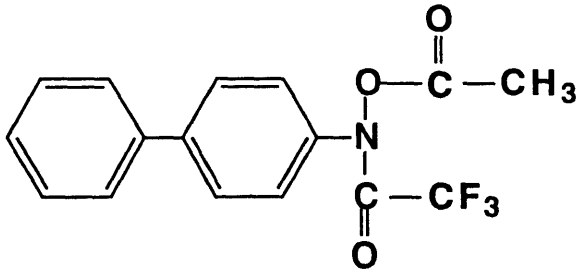
Structures of Reactive ABP Compounds.



N-Hydroxy-4-aminobiphenyl (N-OH-ABP)



N-Acetoxy-4-aminobiphenyl (N-OAc-ABP)



N-Acetoxy-N-(trifluoroacetyl)-4-aminobiphenyl (N-OAc-TFAABP)

The major adduct formed, preliminarily identified as an iminoquinone, was different from those formed from reaction with N-OH-ABP. In addition, *in vivo* studies with bladder explants from humans and dogs have shown that the peroxidase-derived adduct formed predominantly in these tissues (Kadlubar et al., 1988a). However, in dogs given a single dose of ABP, the iminoquinone formed to a minor extent; even after multiple dosing this adduct was apparently not evident.

DNA Adducts of Related Aromatic Amine Compounds

The arylamide of ABP, 4-acetylaminobiphenyl (AABP) forms primarily mammary tumors in rats (Shirai et al., 1981). When the hydroxamic acid of AABP, N-OH-AABP, was reacted with rat liver DNA, the binding level of this compound was considerably lower than the binding of the related aromatic amide, N-hydroxy-2-acetylaminofluorene (N-OH-AAF) (Kriek, 1969; Beland and Kadlubar, 1985). The lower number of adducts formed by AABP could be one explanation for the finding that AAF is a more potent liver carcinogen than AABP (Miller et al., 1961; Beland and Kadlubar, 1985). The DNA adducts formed by AABP have been characterized: 80% of the adducts were determined to be the deacetylated adduct, dG-C8-ABP, and the remaining were the acetylated dG-C8-AABP adduct, and a minor 3-N^2-dG-AABP adduct (Kriek and Westra, 1979; Gupta and Dighe, 1984).

AAF is an extensively studied carcinogen that induces liver tumors in rats (Wilson et al., 1941). In rat liver both the acetylated AAF adduct, *N*-(deoxyguanosin-8-yl)-*N*-acetyl-2-aminofluorene, dG-C8-AAF, and the corresponding deacetylated adduct, *N*-(deoxyguanosin-8-yl)-2-aminofluorene,

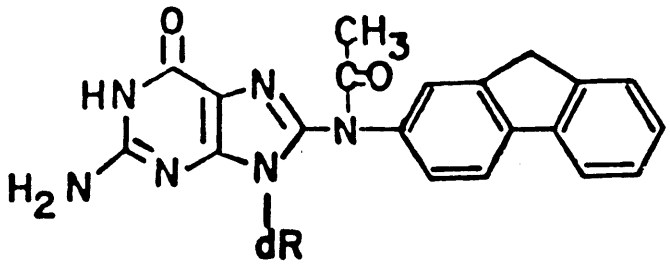
dG-C8-AF, as well as a minor 3-(deoxyguanosin-N^2-yl)-AAF, dG-N^2-AAF, are formed (Figure 6) (Kriek and Westra, 1979). In rats, the deacetylated adduct accounts for 80% of the binding after a single dose of AAF or N-OH-AAF (Kriek, 1969). The acetylated adduct, dG-C8-AAF has a half-life of 7 days, while the deacetylated adduct, dG-C8-ABP was relatively persistent in rat liver DNA (Visser and Westra, 1981). The formation of acetylated adducts of AAF probably arises through the sulfotransferase pathway while the deacetylated adducts form through *N,O* acyltransfer or deacetylation of N-OH-AAF (Miller and Miller, 1981; King and Glowinski, 1983). Reaction of DNA with the ultimate carcinogenic metabolite, *N*-acetoxy-*N*-acetylaminofluorene (AAAF), produces the dG-C8-AAF adduct and the minor dG-N^2-AAF (Miller and Miller, 1981; Beland and Kadlubar, 1985). The reaction of N-OH-AF with DNA predominantly results in formation of the dG-C8-AF adduct (Tang and Lieberman, 1983).

When the related aromatic amine 2-NA is reacted with DNA *in vitro*, three adducts have been detected: a dG-C8-2-NA adduct, a dG-N^2-2-NA adduct, and a dA-N^6-2-NA adduct (Kadlubar et al., 1980). The dG-C8-2-NA was found to be the most persistent adduct in bladder DNA, and, for this reason, some workers have suggested it may be responsible for the carcinogenic effect of 2-NA (Beland and Kadlubar, 1985).

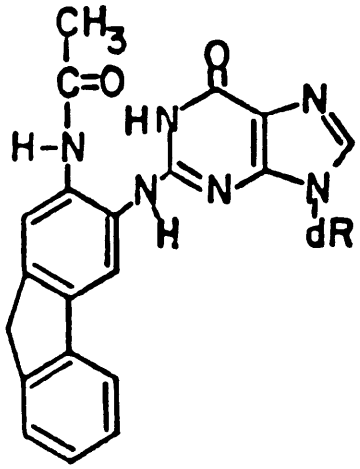
Acetylated BZ derivatives have shown to be *N*-hydroxylated and then *N*-acetylated to form a DNA binding species (Morton et al., 1979; Martin et al., 1982). The major DNA adduct formed in rats given BZ, or ABZ was *N*-(deoxyguanosin-8-yl)-*N'*acetylbenzidine, dG-C8-ABZ. Two major BZ-DNA adducts through the PHS peroxidative metabolic pathway have been identified

Figure 6.

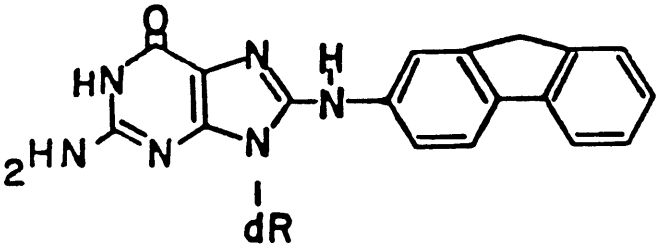
Structures of the DNA adducts of *N*-acetyl-2-aminofluorene.



N-(deoxyguanosin-8-yl)-N-acetyl-2-aminofluorene (dG-C8-AAF)



3-(deoxyguanosin-N^2-yl)-N-acetyl-2-aminofluorene (dG-N^2-AAF)



N-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF)

(Yamazoe et al., 1988). The major one, *N*-deoxyguanosin-8-yl)-benzidine (dG-C8-BZ), and a minor one, *N*,3-(deoxyguanosin-N^7,C^8-yl)-benzidine were detected in incubations of DNA with the peroxidase enzyme.

Aromatic Amines and DNA Interaction Studies

Although a spectrum of carcinogen-DNA adducts can form both *in vivo* and *in vitro* from the reaction of aromatic amines with DNA, it may be the persistence of the unremoved adducts in the DNA, and the nature of the chemical-DNA adduct structure that are the primary determinants of the biological effects of the adducts. The effects of these carcinogen lesions *in vivo* probably depends on changes induced by the adducts in DNA conformation that affect the structure and function of DNA.

As shown in many studies, when ABP binds to DNA the major adduct formed is the dG-C8-ABP adduct. Binding studies of ABP to DNA have shown that the dG-C8-ABP adduct resides in the major groove of DNA where it does not cause much distortion and may not affect the base-pairing properties of the adducted base (Broyde et al., 1985). A later study conducted by Abuaf et al. (1987) has demonstrated that the binding of ABP to DNA induces a slight B-Z transition in a poly (dG-dC) sequence. In general, it has been suggested that ABP-DNA adducts cause minimal perturbation in DNA structure, which may possibly permit these adducts to escape recognition by cellular repair enzymes.

Compounds such as AAF, cause a major local distortion in DNA structure when bound to DNA as the acetylated adduct, dG-C8-AAF (Hingerty and Broyde, 1982). The binding of AAF to poly (dG-dC) DNA induces a transitional

change in the B-DNA conformation to a left-handed Z-DNA structure and rotates the adducted dG-C8-AAF base from an anti to a syn conformation about the *N*-glycosidic bond (Grunberger and Santella, 1981). A recent study done by Koehl et al. (1989), investigated the effect of the position of an AAF-DNA adduct within a DNA sequence that was determined from mutagenesis studies to be a mutation-prone sequence. This study found that the location of the AAF-adduct in the hot-spot sequence affected the local structure of the DNA, inducing it from a B-DNA conformation to a Z-like DNA structure. The results, however, do not correlate with the mutation data within the hot-spot sequence (Burnouf et al., 1989).

In contrast to the acetylated AAF adduct, initial studies indicated that much less of a conformational change was observed to occur with the major AAF adduct formed in DNA *in vivo*, the deacetylated DNA adduct, dG-C8-AF (Broyde and Hingerty, 1983). A more recent study investigated the conformational changes induced in DNA by a dG-C8-AF adduct (Norman et al., 1989). This study found that the dG-C8-AF adduct causes more of a structural distortion in DNA than previously observed. In the sequence in which it was situated, the dG-C8-AF adduct was found to form a more stable base-pair (bp) with deoxyadenosine (dA) than with deoxycytosine (dC). This favorable base-pairing structure was postulated to be responsible for the predominant G -> T mutation formed by this adduct *in vivo* (Bichara and Fuchs, 1985; Carothers et al., 1988).

Another study compared the presence of either an dG-C8-ABP or a dG-C8-AF adduct in a pentadecamer *ras* gene sequence (Marques and Beland, 1990). The results of this study indicated that in this sequence the

structural perturbations induced by these two carcinogen adducts were similar; distortion of the DNA helix was minimal.

It has been suggested that the conformational properties of ABP-DNA adducts compared to AAF or AF adducts may contribute to the similarities and/or differences in the biological consequences of these compounds (Shapiro et al., 1986).

Although ABP adducts form bulky lesions in DNA, if they are not removed by cellular enzymes, they may pose a threat to the replication machinery of the cell and possibly result in mutations during replication. The results of DNA conformation studies have proposed that low energy *syn* conformers of ABP that form due to rotation of the ABP adducted-base-sugar bond may be mutagenic during DNA synthesis at the replication fork (Broyde et al., 1985).

Replication and Repair of Aromatic Amine-Modified DNA

DNA Replication in E. coli

DNA replication in *E. coli* is normally an error-free process, with the error frequencies in the duplication of the *E. coli* genome recorded at 10^{-9} to 10^{-10} per replicated base. The high fidelity of DNA replication is achieved by three steps in the synthesis of DNA: (1) the correct selection and incorporation of the complementary base during 5′-3′ DNA synthesis; (2) 3′-5′ exonucleolytic editing of an incorrectly inserted base at the end of the DNA chain; and (3) the post-replication repair of DNA (Echols and Goodman, 1990).

In *E. coli*, DNA polymerase III holoenzyme is the major enzyme responsible for DNA replication. DNA polymerase I plays a role in the repair of damaged DNA and DNA polymerase II is believed to be involved in the mutagenic response in *E. coli* (Echols and Goodman, 1990). The results of *in vitro* assays have shown that an altered form of pol I, pol I*, may be involved in SOS-induced mutagenesis (Lackey et al., 1982). Mutagenic lesions in DNA can fall in two, but not necessary exclusive, categories: (1) replication-blocking lesions, such as UV photodimers which inhibit DNA replication; and (2) error-promoting lesions, such as alkylated bases which allow replication but promote mispairing of these bases during DNA synthesis (Walker, 1984; Echols and Goodman, 1990).

Nucleotide Excision Repair System of E. coli and its Influence on Aromatic Amine-DNA Adducts

Most bulky, helix-distorting DNA lesions such as those formed by UV radiation and aromatic amines such as AAF are replication-blocking lesions. If they are not repaired by cellular enzymes, they may be lethal to the genome of the cell. In bacteria, the nucleotide excision repair system has been shown to remove a variety of chemical-DNA adducts from DNA (van Houten, 1990). The *E. coli* excision repair system is controlled by three genes: *uvrA*, *uvrB*, and *uvrC* (Grossman and Yeung, 1990). The products of these genes control the initial step of the repair of DNA damaged by UV radiation and bulky chemicals such as AAF. The corresponding repair proteins, UvrA, UvrB and UvrC act together with UvrD (helicase) to excise a

12-13 nucleotide sequence of the damaged region of the DNA (Sancar and Sancar, 1988; Selby and Sancar, 1990).

The most widely studied aromatic amine-DNA adducts have been those formed by AAF. Both the acetylated as well as the deacetylated adducts have been recognized and repaired by the *E. coli* excision repair system (Tang and Lieberman, 1983; Pierce et al., 1989; Seeberg and Fuchs, 1990). The rates of removal of the acetylated adducts have been faster than that of the deacetylated adducts probably owing to structural differences in DNA perturbations induced by these adducts.

The availability of *E. coli* strains which are proficient, *uvr*$^+$, or deficient, *uvr*$^-$, in the nucleotide excision repair functions has enabled workers to study the repair and mutagenesis of bulky adducts such as UV lesions and AAF-DNA adducts in *E. coli* (Howard-Flanders et al., 1966).

In Vitro Replication Assays on Aromatic Amine-modified Templates

DNA replication assays conducted *in vitro* with both AAF and AF adducts have shown that the AAF adducts block or terminate DNA synthesis by DNA polymerases (Moore et al., 1980; Fuchs et al., 1983; Sahm et al., 1989). Experiments with double-stranded DNA modified with AAAF have shown that DNA adducts formed by this compound block DNA synthesis at the replication fork where the adducted-DNA is single-stranded, thus probably resulting in lethality or mutation at that site (Armier et al., 1988).

In contrast, accurate translesion synthesis of the AF adduct predominantly occurs during *in vitro* synthesis on AF-modified templates (Michaels et al., 1987; Strauss and Wang, 1990; Michaels et al., 1991).

Other studies have observed mutations formed during *in vitro* assays with AF-modified templates (Sahm et al., 1989). The majority of the mutations were -1 deletion of G's and G -> T transversions at putative dG-AF adducts.

Mechanisms of Mutagenesis by Chemical-DNA Adducts

Mutations in DNA can occur as a result of mispairing of modified DNA bases during DNA replication or ineffective repair of DNA adducts prior to or during DNA synthesis. An incorrect purine-pyrimidine bp can lead to transition mutations, while a purine-purine bp can lead to a transversion mutation (Topal and Fresco, 1976; Topal, 1988). These incorrect base pairs can form through both chemical as well as structural perturbations of the DNA (Loechler, 1988).

Mutations that occur during DNA replication could arise not only from mispairing of adducted DNA bases but also from structural changes in the normal base-sugar conformation induced by DNA adducts. A change in conformation from anti to syn about the *N*-glycosidic bond between the base and the deoxyribose moiety can permit mispairing of G_{syn} with the imino tautomeric forms of either A or G, or mispairing of A_{syn} with the imino tautomers of either G or A, thus resulting in mutations (Topal and Fresco, 1976; Swenson and Kadlubar, 1981). Speculations about the mispairing possibilities of aromatic amine-adducted bases have led to predictions that C8 and N^2-substituted guanines can result in G -> T or G -> C transversions and C8-substituted adenines may cause A -> C or A -> T transversions (Beland and Kadlubar, 1985; Loechler, 1988). In *E. coli*, most bulky adducts formed by aromatic amines such as ABP at dG bases are repaired

efficiently by the cellular repair systems; however, unrepaired adducts could lead to mutations during DNA replication. Many studies have been carried out in *E. coli* investigating the mutagenic specificity as well as the mutational specificity of the DNA adducts formed by carcinogens in these cells (Miller, 1983).

The SOS System of E. coli

E. coli has two major independent regulatory systems than can be induced in response to DNA damage: (1) the SOS system which is controlled by the RecA and LexA proteins, and (2) the adaptive response controlled by the Ada protein (Walker, 1984). In *E. coli*, the first evidence of a cellular response to damaging agents was provided in a study conducted by Weigle (1953). The results of the experiment indicated that exposure of the host bacterial cell to a low fluence of UV radiation prior to infection with a UV-irradiated bacteriophage not only led to a substantial increase in the survival of the UV-damaged phage but was required for a high level of phage mutagenesis. This cellular phenomenon was later found to be inducible by UV irradiation of *E. coli* and was responsible for both the mutagenic reactivation of UV-irradiated phage and for error-prone repair of bacterial DNA (Defais et al., 1971). The cellular phenomenon was eventually promoted as the "SOS hypothesis" by Radman (1975).

The designation "SOS" suggested that damage to bacterial DNA or the inability to replicate DNA initiated a signal that caused the derepression of various gene functions all of which led to the enhanced likelihood of survival for the cell or its phages (Witkin, 1976). The hypothesized

mechanism of the SOS mutagenic pathway is that DNA replication is inhibited by lesions in the template strand so that a base cannot be added opposite the lesion during DNA synthesis (Strauss, 1989). As result of the induction of the SOS gene products, DNA polymerase activity is altered so that synthesis past the lesion occurs. This increased processivity comes with a cost, however, in that there is an increase in the frequency of mutations (Strauss, 1989).

The induction of the SOS response leads to various cellular repair and mutagenic activities. The functions involve different types of DNA repair, including postreplication recombinational repair, double strand break repair, and long patch excision repair. The SOS response alters various aspects of DNA replication, the most important of which is allowing the replication complex to insert nucleotides opposite noncoding template lesions known as the UmuDC-dependent error-prone translesion activity which is responsible for SOS mutagenesis (Walker, 1984; Witkin, 1991).

Mutagenesis by UV lesions or bulky chemical adducts in *E. coli* is dependent on the SOS error-prone system. The SOS response consists of the derepression of approximately 20 *din* or "damage-inducible" genes that are regulated by the RecA and LexA proteins. The expression of these genes are important in the excision-repair of bulky adducts (*uvrA*, *B*, and *C* gene products) and the mutagenic processing of damaged DNA (functions of the *umuDC* operon) (Walker, 1985).

SOS induction and mutagenesis is comprised of two RecA-mediated pathways of translesion synthesis and replication restart, both of which involve alterations in DNA replication mediated by RecA and the UmuC-UmuD'

proteins (Echols and Goodman, 1990). Recently much progress has been made to elucidate the role of the various proteins in the regulation and expression of the SOS response. The major regulator in the SOS system appears to be the RecA protein, which is now presumed to have three roles in SOS mutagenesis (Echols and Goodman, 1990).

The first role of RecA is in regulation of the expression of the SOS genes. It is believed that DNA damage generates a signal, presumably single-stranded regions of DNA, to which RecA binds along with another cofactor, probably dATP. This binding complex results in an altered form of the RecA protein designated RecA*. RecA* facilitates the proteolytic autocleavage of the LexA protein, which is the repressor of the genes of the SOS regulon, including the *recA* gene itself as well as the *umuDC* genes (Sweasy et al. 1990). When DNA repair is completed or replication of DNA is resumed, RecA is no longer activated, LexA is no longer cleaved and the repression of the SOS genes is restored (Witkin, 1991). Recent evidence suggests that the UmuD protein may also be important in the decay of the SOS response (Battista et al., 1990b).

In its second role, RecA protein has been shown to activate proteins by facilitating the autocleavage of the UmuD protein to the form, UmuD', which is the form active in SOS mutagenesis (Burckhardt et al., 1988; Nohmi et al., 1988; Shinagawa et al., 1988). Only UmuD' has been shown to be functional in SOS-induced mutagenesis (Nohmi et al., 1988). One current hypothesis is that UmuC and UmuD' form a complex and may facilitate elongation of DNA by polymerase III past a DNA lesion (Battista et al., 1990a). Recent evidence suggests that the intact UmuD protein is a

negative regulator of its cleaved product, the UmuD' protein (Battista et al., 1990b). These workers demonstrated that UmuD-UmuD' heterodimers form *in vitro* and postulated that these complexes lead to the decline of the SOS response in *E. coli*.

A third direct role for RecA in SOS mutagenesis has been hypothesized (Battista et al., 1990a; Sweasy et al., 1990). Studies have indicated that RecA may inhibit the 3'-5' proofreading exonuclease of the epsilon subunit of the DNA polymerase III complex (Lu et al., 1986; Witkin, 1991). Two possible mechanisms have been suggested for the role of RecA in SOS mutagenesis (Echols and Goodman, 1990). RecA may be involved in translesion mutagenic replication, or replicative bypass I, in which RecA protein binds to a DNA lesion facilitating polymerase III bypass of the damaged base. Alternatively, RecA may be involved in replication restart, or replicative bypass II, in which pol III might bypass the lesion by copying the undamaged strand (Dutriex et al., 1989; Woodgate et al., 1989). Elucidation of the biochemical mechanisms of RecA, UmuD and UmuC in SOS mutagenesis using purified proteins remains to be established.

Analogues of the *umuC* and *umuD* mutagenic processing genes of *E. coli* have been found in naturally occurring plasmids found in bacteria. A derivative of the *Salmonella typhimurium* plasmid R46, plasmid pKM101, which encodes the *mucA* and *mucB* genes, has been used in the Ames assay to enhance the susceptibility of cells to mutagenesis (McCann et al., 1975; Walker, 1978). The products of the *mucA* and *mucB* genes are important in the mutagenesis resulting from bulky DNA adducts in bacteria (Walker, 1984).

Plasmid pGW16, a mutant of pKM101, has been shown to induce high mutation rates in *E. coli* (Walker, 1978; Lasko et al., 1988; Little et al., 1991).

Mutagenesis Studies with Aromatic Amines in Bacteria

The early mutagenesis studies with aromatic amines were carried out in *S. typhimurium*, a strain that is sensitive to aromatic amines that are carcinogenic for humans and animals. Subsequently, mutation assays were conducted in *E. coli* to evaluate the genotoxicity of a wide range of chemicals. The bacterial studies were then used as models to develop mutagenesis studies with mammalian cell culture systems.

Mutagenesis Assays in S. typhimurium

The initial *S. typhimurium* mutation assays were developed in the laboratory of Ames and colleagues (Ames et al., 1975; McCann et al., 1975). Within a short time the investigators had demonstrated that 85% of the carcinogens tested were found to be bacterial mutagens in their experimental system.

The mutagenicity of ABP and related aromatic amines has been investigated using the *S. typhimurium* assays (McCann et al., 1975). In these assays the *his* gene of *S. typhimurium* is used as the target for mutation. The *S. typhimurium* strains used are described as follows: TA1538 is a frameshift tester strain; its derivative, TA98, harbors the plasmid pKM101. TA1535, another tester strain, is used to detect bp substitution mutations at a G-C bp target and TA100 is a derivative of this strain containing pKM101 (Maron and Ames, 1984). The presence of pKM101 in

S. typhimurium tester strains has been observed to enhance the SOS-dependent mutagenesis by various chemicals (McCann et al., 1975; Walker, 1978).

Either ABP in the presence of a metabolic activating system (liver S-9 fraction), or N-OH-ABP caused mutations in *S. typhimurium* strains TA1538, TA98 and TA100, but not TA1535 (El-Bayoumy et al., 1981; Beland et al., 1983). Mutagenesis by ABP was highest towards TA98 with the S-9 activating system and AF was found to be mutagenic in TA98, TA1538 and TA100 (Scribner et al., 1979). Connor and coworkers (1983) found ABP, AF and AAF to be mutagenic in TA98 with the S-9 enzyme fraction with the order of mutagenicity being AF > AAF > ABP > AABP. In TA100, AF was approximately equivalent to ABP.

For BZ, either *N*-acetylation or peroxidative metabolism could produce reactive metabolites that were mutagenic in bacteria (De France and Josephy, 1987). The requirement for acetylation enzymes in the metabolic activation of BZ was demonstrated using the *S. typhimurium*/hepatocyte assay (Neis et al., 1984). In this study BZ was found to be mutagenic in the *S. typhimurium*/rat hepatocyte system; however, ABP was more mutagenic with *S. typhimurium* and only with the S-9 enzyme fraction from hepatocytes. Acetylation is a metabolically activating step in BZ metabolism; however, it is a detoxification step in ABP metabolism (Kadlubar et al., 1982b; Hein 1988).

In assays conducted with *S. typhimurium* TA104, a strain carrying pKM101, which can be used to detect mutations at an A-T bp target (Levin et al., 1984), ABP treatment resulted in increased reversion to *his*

prototrophy (Lasko et al., 1988). Together, the results from the *S. typhimurium* studies demonstrate that mutagenesis by ABP and related aromatic amines is enhanced in the presence of SOS-processing functions with frameshift mutations and base-pair substitution mutations occurring at both G-C and A-T bp targets.

Mutagenesis Studies in E. coli

The mutagenicity of ABP and related aromatic amines has also been studied using various mutation assays in *E. coli*. N-OH-ABP was observed to be a direct-acting mutagen in *E. coli* WP2*uvrA*, an excision-repair deficient strain. An increased level of mutation was observed in the same cells that contained the plasmid pKM101 (Pai et al., 1985).

A recent study investigated AAF mutagenesis directly in the *lacI* gene of *E. coli* (Schaaper et al., 1990). These investigators treated bacterial cells with AAAF and analyzed the mutations formed *in vivo*. The mutations were found to be a mixture of both base-substitution and frameshifts, suggesting that the mutations formed probably arise from both the acetylated (AAF) adducts as well as the deacetylated (AF) adducts. The latter was suggested to be formed from deacetylation of AAAF in the bacterium prior to reaction with DNA (Schaaper et al., 1990).

Mutagenesis Assays Using Plasmid or Viral-Based Vectors

Extensive studies have been conducted with aromatic amines using plasmid or viral-based vector systems in mutagenesis assays in *E. coli*. This section will first review the biology of M13 bacteriophage, the vector

used in viral-based assays reported in this dissertation as well as in many other studies. Subsequently, mutagenesis studies in *E. coli* with aromatic amine-modified M13 vectors or extrachromosomal plasmids modified with aromatic amines will be reviewed.

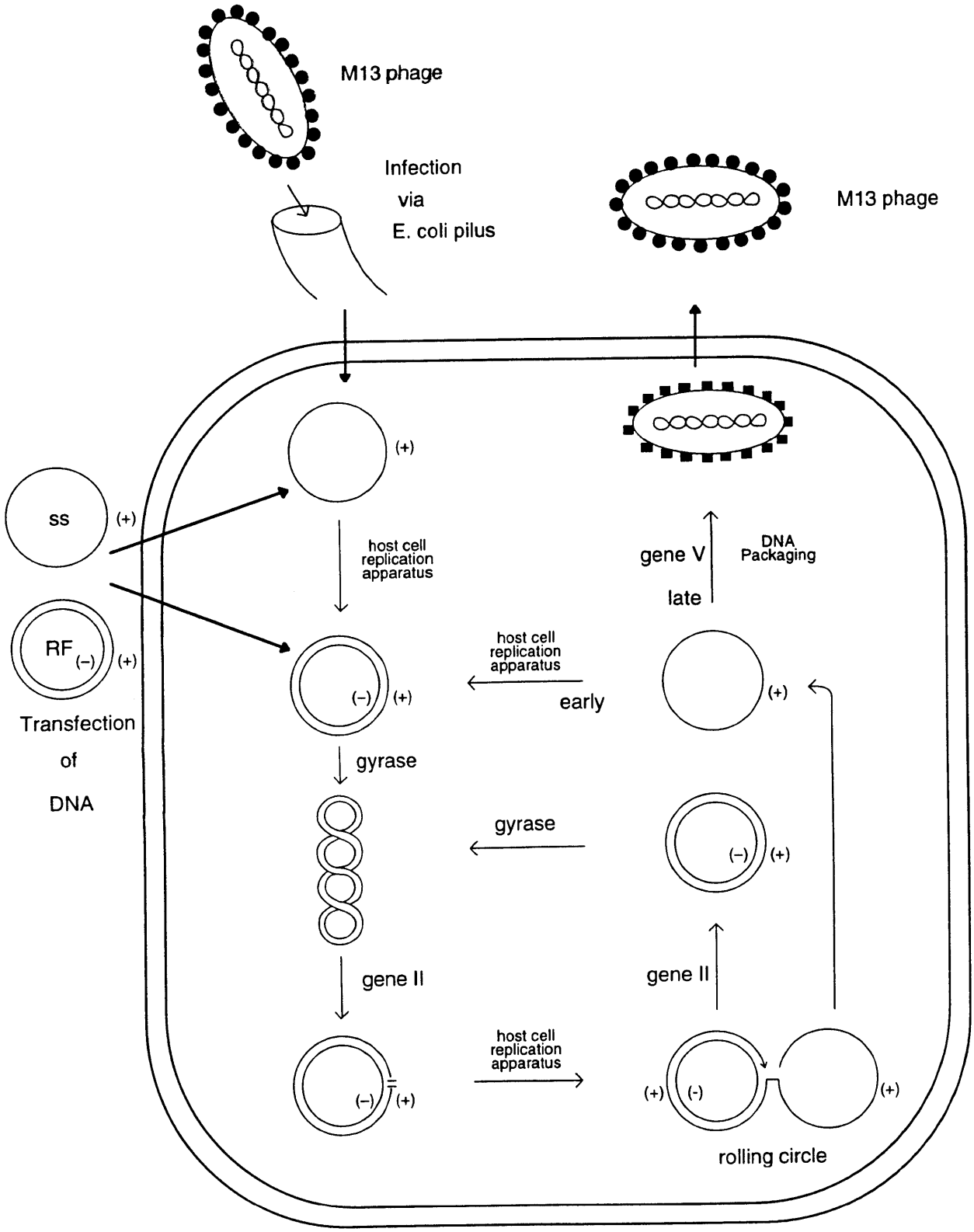
The M13 Bacteriophage Experimental System

M13 Life Cycle

M13 is a single-stranded filamentous bacteriophage that infects *E. coli* cells containing an F factor plasmid (Figure 7). M13 adsorbs to the F pilus on male bacteria and releases its DNA into the cytoplasm of the cell. After infection, once the single-stranded viral DNA (the plus strand) is inside the cell, the complementary strand (the minus strand) is synthesized using the host cell replication enzymes to form the duplex replicative form (RF) DNA. After the RF is formed, M13 protein synthesis takes place. The M13 genome encodes ten essential genes required for phage viability as well as an intergenic region (IR), which bears the origins of replication. The RF DNA is converted to viral DNA by the rolling circle method of replication after M13 gene II protein nicks the plus strand of the duplex. When the intracellular pool of RF molecules has accumulated to about 100-200 copies, DNA synthesis is focused on producing viral strands. The M13 gene V protein sequesters the newly synthesized viral strands and together with M13 gene VIII product, which forms the major coat protein of the bacteriophage, the viral DNA is packaged, and extruded out of the cell as phage particles (Ray, 1978; Rasched and Oberer, 1986). M13 bacteriophage does not lyse its host but infected cells grow more slowly

Figure 7.

The Life Cycle of Bacteriophage M13. Phage infection of an M13 host strain occurs via the pilus. Alternatively, M13 ss or RF DNA can be taken up by the cell via a calcium chloride/heat shock transfection procedure. Details are described in text (adapted from Bradley, 1991).


M13 phage
Infection
via
E. coli pilus
M13 phage
ss
(+)
RF
(–)
(+)
Transfection
of
DNA
host cell
replication
apparatus
gene V
late
DNA
Packaging
host cell
replication
apparatus
early
gyrase
gyrase
gene II
gene II
host cell
replication
apparatus
rolling circle

E. coli **cell**

than uninfected cells. Infectivity with M13 can be high, leading to a phage titre of 10^{12} phage particles or plaque-forming units/mL (PFU/mL) of bacterial culture (Messing, 1983).

M13 as a Cloning Vector

The M13 RF genome has been modified to form cloning vectors (the M13mp series) that contain within the IR region, a portion of the *lac* gene regulatory system (operator and promoter), a part of the *lacZ* gene (the α fragment) and a series of unique restriction sites that can be used for insertion of DNA fragments (Messing et al., 1977) (Figure 8). When M13mp phage, M13 ss or RF DNA is inside an appropriate host cell (*lacZ* ΔM15), α-complementation can occur between the *lacZ*α gene in the M13 genome and the *lacZ* ΔM15 deletion of the *lacZ* gene carried on an F episome (F') in the host bacterium (Langley et al., 1975; Welply et al., 1981). The *lac* operon can be induced by IPTG (isopropyl-β-D-thiogalactopyranoside), a synthetic inducer that binds the Lac repressor, thus permitting transcription of *lacZ*α mRNA from M13 RF DNA. The gene product of the α gene fragment, the NH_2 portion of the protein, complements the COOH portion of the protein produced from the ω fragment of the *lacZ* gene on the F' in the host cell. This results in expression of a biologically active protein, β-galactosidase, an enzyme that cleaves lactose or a synthetic substrate such as X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) into its respective subunits (Messing, 1983). Normal beta-galactosidase production results in cleavage of a chromogenic substrate such as X-gal (an indicator

Figure 8.

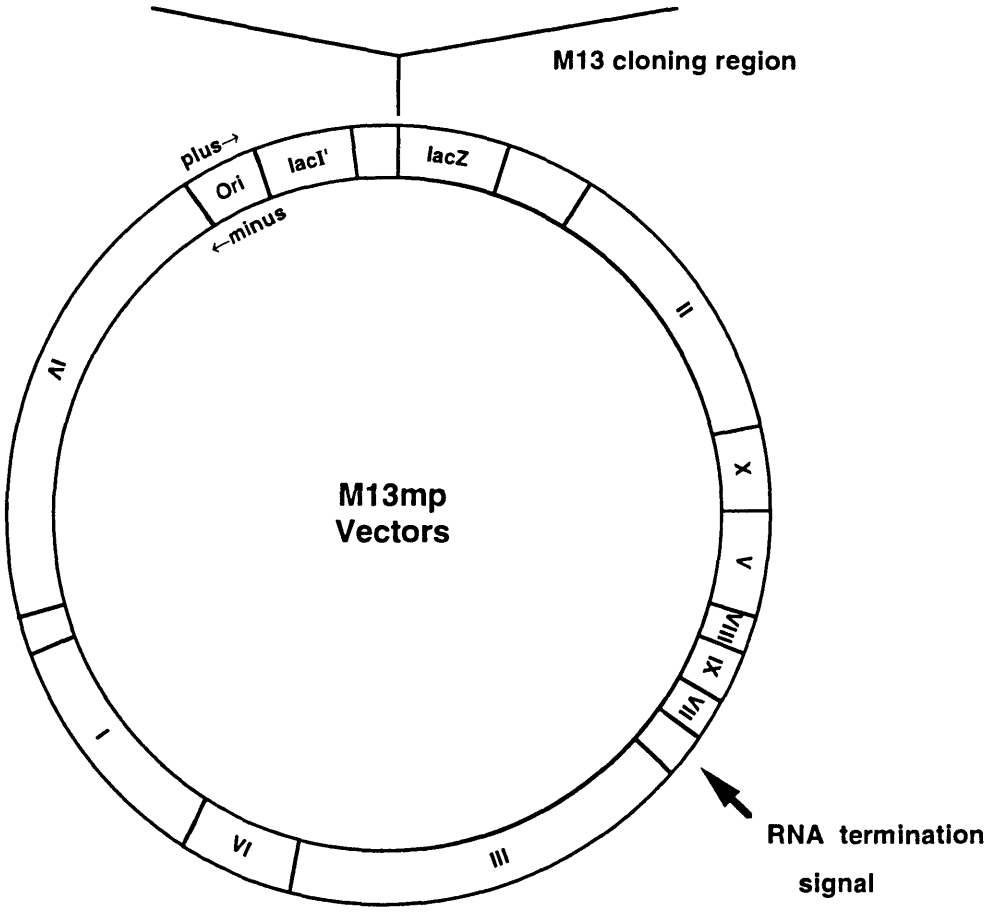
The M13 Cloning Vectors used in the study of 4-aminobiphenyl mutagenesis in *E. coli*. M13mp10 single-stranded and double-stranded DNA were used in the random mutagenesis experiments. Two derivatives of M13mp19+ double-stranded DNA were used in the site-specific mutagenesis experiments.

M13mp10 G AAT TCG AGC TCG CCC GGG GAT CCT CTA GAG TCG ACC TGC AGC CCA AGC TT

Eco RI Sma I Bam HI Pst I Hind III

M13mp19+ ACC ATG ATT ACG CCA AGC TTG CCC TGC AGG GCG AAT TCA CTG GCC GTC GTT

Hind III Pst I Eco RI



dye) to yield an indigo compound that produces blue plaques on an indicator plate containing M13 host cells, X-gal and IPTG.

The M13 lacZ Gene Mutation Assay

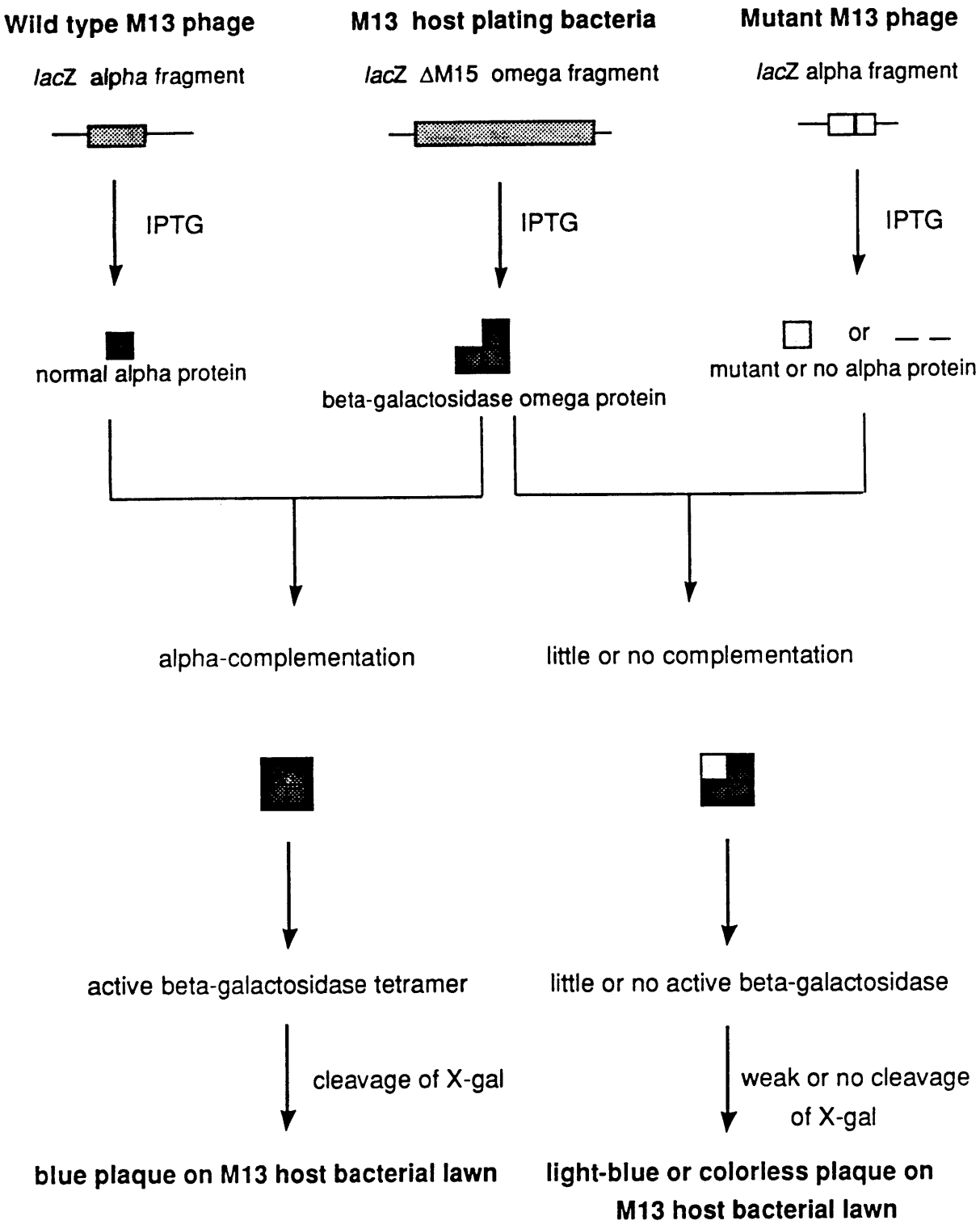
The M13 phage genome can be used as a facile and useful system for mutagenesis studies (Kunkel, 1984; Loechler et al., 1984). The *lacZα* gene in the M13mp genome serves as the target for mutation in this system. M13 phage DNA (ss DNA) or M13 RF DNA (double-stranded or ds DNA) can be treated with chemical agents, introduced into host cells, replicated *in vivo* and then analyzed for mutations. When M13 is plated on appropriate host cells, such as *E. coli* GW5100 (*lacZ* ΔM15), X-gal and IPTG, plaques with a blue phenotype (wild-type) are produced after infection by M13 phage. Mutations in the *lacZα* gene that affect the biological properties of β-galactosidase result in proteins that are either slow to cleave X-gal, producing light-blue plaques, or inactive proteins that yield colorless plaques on indicator plates (Figure 9).

The *lacZ* forward mutation assay has been used to evaluate the mutagenicity of ABP-DNA adducts (Lasko et al., 1988); AF-DNA adducts (Gupta et al., 1988) and AAF-DNA adducts (Gupta et al., 1991) in the *lacZα* gene of randomly modified M13 ss or ds genomes. This forward mutation assay can be used to detect many bp substitution mutations as well most insertions and deletions (Kunkel et al., 1984).

For site-specific mutagenesis assays, DNA adducts of ABP (Lasko et al., 1988), AF and AAF (Reid et al., 1990) have been situated in the cloning region of the *lacZ* gene at a site where bp substitution mutations should

Figure 9.

The M13 *lacZα* gene Mutation Assay. The β-galactosidase alpha protein synthesized from the *lacZ* gene of the M13 genome complements the β-galactosidase omega protein produced from the M13 host bacterial cell to form an active β-galactosidase polypeptide. Four β-galactosidase polypeptides aggregate into a tetramer to form an active β-galactosidase enzyme (adapted from Lasko, 1986).


Wild type M13 phage
lacZ alpha fragment
IPTG
normal alpha protein
M13 host plating bacteria
lacZ ΔM15 omega fragment
IPTG
beta-galactosidase omega protein
Mutant M13 phage
lacZ alpha fragment
IPTG
or
mutant or no alpha protein
alpha-complementation
little or no complementation
active beta-galactosidase tetramer
cleavage of X-gal
blue plaque on M13 host bacterial lawn
little or no active beta-galactosidase
weak or no cleavage of X-gal
light-blue or colorless plaque on M13 host bacterial lawn

not disrupt normal production of β-galactosidase; however, insertions, deletions or in-frame termination codons would affect the normal expression of the *lacZ* gene.

Mutagenesis Studies with Aromatic Amine-Modified DNA in *E. coli*

Assays with Globally-Modified Vectors

The mutational spectrum of ABP and the survival of ABP-modified M13mp10 RF DNA in *E. coli* have been assayed in a random mutagenesis experiment conducted in our laboratory (Lasko et al., 1988). This study demonstrated that survival of ABP-modified M13mp10 duplex genomes in excision-repair proficient strains (*uvr*$^+$) was higher than in excision-repair deficient cells (*uvrA* or *uvrC*). When M13 ds DNA, randomly adducted with ABP, was transfected into *uvr*$^+$ cells containing pGW16, there was a 40-fold increase in mutagenesis above background in an SOS-processing environment. The primary mutations observed in the *lacZ* gene were found at dG sites with G:C -> T:A and the G:C -> A:T mutations representing the majority of the sequence changes (Lasko et al., 1988).

A later study compared the genotoxic effects of DNA adducts formed by AABP and ABP to DNA adducts formed by AAF and AF in a plasmid pBR322 assay system (Tamura and King, 1990). These workers were investigating the influence of the excision repair of deacetylated and acetylated DNA adducts on plasmid survival. The results indicated that there was no significant difference in the genotoxic effects between AABP and ABP on the survival of adducted plasmid DNA in *E. coli*. In addition, AABP-DNA adducts were as

genotoxic as ABP-adducted plasmid DNA in *uvr*$^+$ strains compared to *uvr*$^-$ strains. In contrast, the AAF-DNA adducts were more genotoxic than the AF-DNA adducts in plasmids replicated in either *uvr*$^+$ or *uvr*$^-$ strains. The authors postulated that structural differences between AABP and AAF adducts were responsible for the differences in genotoxicity induced by the two adducts.

In studies done with reactive metabolites of AAF, an AAAF-treated plasmid was demonstrated to be highly mutagenic in *E. coli* (Koffel-Schwartz et al., 1984). The reaction of the ultimate carcinogen, AAAF, with DNA produces primarily acetylated dG-C8-AAF adducts. The majority of mutations induced by this carcinogen were SOS-dependent frameshifts in the tetracycline gene of pBR322. These mutations fell into two categories: -1 frameshifts at repetitive dG sequences; and -2 G-C frameshift mutations at *Nar* I, 5'-GGCGCC-3' sequences, which were *umuDC*-independent (Koffel-Schwartz et al., 1984). In addition, there was no direct association between the preferential sites of AAF-DNA modification and the AAF mutation spectrum (Fuchs, 1983). The results suggest that there are mutation-prone sequences in which the binding of the carcinogen to the DNA may induce a conformational change such that the lesion becomes a target for the enzymes or proteins involved in the processing of the lesion into a mutation (Bichara and Fuchs, 1985). A recent study using an AAAF-modified M13 double-stranded vector showed that AAF adducts induced both -1 G frameshifts and various bp substitution mutations in the *lacZ* gene target of M13 with approximately equal frequency (Gupta et al., 1991). The authors suggest that differences in the types of vectors used in the

mutation assays as well as the different mutational targets may contribute to the differences in the observed mutations from the plasmid system of Fuchs and coworkers (Koffel-Schwartz et al., 1984).

By contrast, mutations caused by the reactive AF metabolite, N-OH-AF, which forms primarily the deacetylated adduct, dG-C8-AF, in a plasmid vector were predominantly G:C -> T:A bp substitution mutations (Bichara and Fuchs, 1985). Mutagenesis by AF was shown to be dependent on the SOS *umuDC* gene functions of *E. coli*. The requirement for SOS induction for a significant increase in mutation frequency with an N-OH-AF treated M13mp10 duplex genome was demonstrated in a study conducted by Ross et al. (1988). The results of this study showed that without prior SOS induction of the host cell, mutation frequency of the adducted DNA did not significantly increase above background. Another study compared N-OH-AF mutagenesis induced in M13mp8 ss and ds DNA (Gupta et al., 1988). In this assay, the mutation frequency of M13 ss DNA modified with N-OH-AF increased 10-fold above background in SOS-induced cells. In AF-modified ds DNA the increase in mutation frequency was less than in ss DNA. Presumably, adducts in ss DNA are more mutagenic than adducts in ds DNA owing to the increased capacity for ds DNA repair by the host cellular enzymes (Lindahl, 1979).

Differences in the types of DNA-adducts formed by different aromatic amines and the alterations in DNA structure induced by these aromatic compounds may account for the differences in mutagenicity of these compounds and types of mutations observed in bacterial test systems. Together, the results from experiments conducted in *E. coli* suggest that the gene products of the SOS system are important for the mutagenicity of

DNA adducts formed by ABP and related aromatic amines as well as for the survival of aromatic amine-modified ss and ds genomes. The results from random mutagenesis experiments have been invaluable in the data they have provided regarding both the mutation frequency induced by various aromatic amines in similar assay systems as well as the mutational specificity of the different aromatic amine compounds.

Although the mutational spectrum induced by a carcinogen is informational, it is often difficult to assign a particular mutational change to a specific carcinogen-DNA lesion. Advances made in the field of single-lesion mutagenesis have enabled investigators to make hypothesis about individual chemical-DNA adducts and test these hypothesis in the same plasmid and viral systems used to study random mutagenesis induced by these chemical carcinogens (Wood and Essigmann, 1991).

Assays with Site-Specifically Modified Vectors

It recently has become possible to study the biological effects of a single DNA lesion situated in a vector that is replicated *in vivo*. Early work in this field was conducted in *E. coli*, a host cell that is easy to propagate and manipulate in culture. These accomplishments were made possible by, (1) the availability of plasmids and viral genomes that could be easily genetically engineered for the study of single-lesion mutagenesis and (2) advances in the chemical synthesis of deoxyoligonucleotides containing single chemical-DNA adducts (Basu and Essigmann, 1988).

Various methods have been described to construct genomes containing single chemical-DNA lesions (Singer and Essigmann, 1991). One method

involves ligating a site-specifically modified oligonucleotide into a gapped duplex viral genome to create a singly-adducted vector (Green et al., 1984; Naser et al., 1988). A second technique utilizes a primer extension method to create a genome from a modified oligonucleotide annealed to viral or plasmid genome (Chambers et al., 1985; Kodadek and Gamper, 1988). The first relies on the placement of the adduct within a restriction site in the genome, whereas the second method can be used to situate the adduct at almost any site in the genome.

Initially, DNA adducts induced by alkylating agents were studied because of the ease in which they could be synthesized and situated in genetic vectors. The DNA adduct, O^6-methlyguanine, is a minor product formed by alkylating agents in DNA. The initial site-specific experiments investigating the mutagenicity of the O^6-methlyguanine adduct were conducted by Loechler et al. (1984) and were followed by a number of studies that employed different methods for studying *in vivo* mutagenesis induced by alkyl-DNA adducts in plasmid or viral genomes (Chambers et al., 1985; Bhanot and Ray, 1986; Hill-Perkins et al., 1986; Ellison et al., 1989; Klein et al., 1990; Dosanjh et al., 1991).

The possible mutagenic roles of the specific DNA adducts of aromatic amines and amides have also been extensively studied in *E. coli* (Lasko et al., 1988; Moriya et al., 1988; Burnouf et al., 1989; Reid et al. 1990). In a site-specific mutagenesis experiment, a single dG-C8-ABP adduct, when present in a double-stranded M13mp10 genome, was observed to be not mutagenic even though ABP was mutagenic in the same duplex genome in random mutagenesis experiments (Lasko et al., 1988). A bias against replication

of the ABP-adducted strand of the singly-modified vector was suggested as one explanation for this unexpected observation. In fact, this hypothesis has been promoted based on a study conducted with a ds plasmid modified with AAAF (Koffel-Schwartz et al., 1987). The investigators reported a profound replication bias *towards* the unadducted strand when DNA adducts were present on only one strand of a ds plasmid. The results of this study are important because it suggests that adducts present in one strand of duplex DNA may be rapidly repaired or bypassed *in vivo* and hence these data may explain our observation of little or no mutagenesis in the site-specific mutagenesis study (Lasko et al. 1988).

After these experiments were published, other results from our laboratory found that adducts in ss DNA were more mutagenic than in ds DNA (Basu et al., 1988; Bradley, 1991). To circumvent the dilemma of studying single lesions in duplex vectors, researchers have placed UV lesions or put uracil residues in DNA to inactivate one strand of a duplex genome or plasmid vector thereby forcing replication onto the adducted strand during *in vivo* synthesis of the singly-adducted vector (Burnouf et al., 1989; Reid et al., 1990). Studies conducted in our laboratory have found that the use of bacteriophage M13 is advantageous in that the ds as well as the ss DNA forms of this bacteriophage can be manipulated and used to study single-lesion mutagenesis (Basu et al., 1988; Lasko et al., 1988; Wood et al., 1990).

Site-specific mutagenesis experiments with AAF adducts have investigated the effect of a single dG-AAF adduct in a mutation prone sequence in a plasmid vector replicated in bacterial cells (Burnouf et al.,

1989). The position of the adduct within the 5'-GGCGCC-3' sequence significantly contributed to the mutagenicity of the lesion *in vivo*. The results observed from this investigation indicated that mutagenesis induced by the adduct was SOS-dependent and the primary mutational change was a -2 frameshift, which is consistent with the mutational spectrum of AAF observed by this same group (Koffel-Schwartz et al., 1984).

Another laboratory has investigated the genotoxic effect of either the dG-C8-AAF or the dG-C8-AF adduct in an M13 vector replicated in bacterial cells (Romano et al., 1988; Gupta et al., 1989; Reid et al., 1990). Although the investigators report that the mutation frequency was increased due to the presence of the adduct, most of the actual mutations observed were not targeted to the site of the lesion in the vector (Gupta et al., 1989). In fact the AAF adduct was found to be less mutagenic than the AF adduct. The latter was found to induce -1 frameshift mutations in ds DNA and bp substitutions in ds DNA containing uracil residues in the strand opposite the adduct (Reid et al., 1990). In addition, the induced mutations were observed to be different in SOS-induced and non-SOS-induced backgrounds. An earlier study investigating a single dG-C8-AF adduct in M13 DNA had also noted that this lesion induces -1 deletion mutations, albeit in a non-SOS dependent manner (Mitchell and Stohrer, 1986). The results of these studies do not correlate with the random mutagenesis experiments with AF-modified DNA (Bichara and Fuchs, 1985). Differences in the experimental systems used to assay the AF- and AAF-adducted genomes may explain the different results observed by these groups (Burnouf et al., 1989; Reid et al., 1990).

While the assays using plasmid and viral-based assays were being conducted, plasmids referred to as shuttle vectors were being developed to study mutagenesis in both bacterial and mammalian cells. One of the first studies investigating aromatic amine-DNA adducts using shuttle vectors was conducted by Moriya et al. (1988). These workers found that a single dG-C8-AAF lesion situated in a duplex vector was mutagenic at a high frequency when assayed in bacteria without SOS-induction; the primary mutation observed was the G -> C transversion. Once again, however, the type of mutation observed is not what was expected based upon the mutational spectrum induced by AAF in bacteria (Koffel-Schwartz et al., 1984).

Mutagenesis Studies with Aromatic Amines in Mammalian Cells

Assays with Endogenous Genes of Mammalian Cells

Mutagenesis studies have also been conducted in mammalian cells with aromatic amine-modified shuttle vectors as well in the endogenous genes of mammalian cells in culture.

In mammalian cells, ABP, AAF and BZ in the presence of an activating liver S-9 fraction, were found to induced $TK^{-}/^{-}$ mutations in the L5178Y$TK^{+}/^{-}$ mouse lymphoma forward mutation assay (Oberly et al., 1984). AAF was demonstrated to be mutagenic in the dihydrofolate reductase gene of cultured Chinese hamster ovary cells (Carothers et al., 1988). The majority of the induced mutations were base-substitutions in the *dhfr* gene. The investigators found that the majority of the adducts that were analyzed

after treatment of CHO cells *in vivo* with AAF were the deacetylated, dG-C8-AF adducts (Carothers et al., 1988).

Although the AAF results in mammalian cells do not agree with the results observed in bacteria (Koffel-Schwartz et al., 1984), they are consistent with the theory that mammalian cells may possess deacetylase enzymes that remove the acetyl group from AAF prior to reaction with DNA or perhaps after adduction with DNA. This theory has been offered as an explanation for the results observed in an early study conducted by Moriya et al. (1988), in which a site-specifically dG-C8-AAF modified shuttle vector was replicated in COS-1 monkey cells with a high mutation frequency. The mutational changes induced by the lesion were G -> C and G -> T transversions that occurred with approximately equal frequency. Although, *N*-acetyltransferases that catalyze *N*,*O*-acyltransfer of acetylated compounds have been detected in mammalian cells (Mattano et al., 1988), and could explain the observations of Carothers et al. (1988); there is no experimental evidence, however, to support the existence of an adduct deacetylase.

Assays with Aromatic Amine-Modified Shuttle Vectors

The mutagenic effects of AAF and AF have been extensively studied in shuttle vector systems by Maher and colleagues (Maher et al., 1989; Mah et al., 1989; Mah et al., 1991). The mutations produced by an AF-modified plasmid replicated in a human kidney cell line were primarily base-substitution mutations, with G:C -> T:A being the predominant mutation (Mah et al., 1989). A recent study conducted by this group compared mutagenic

efficiencies between AAF and AF adducts in a shuttle vector replicated in human cells (Mah et al., 1991). The results indicated a similar mutation spectrum for both AAF and AF adducts, predominantly bp substitutions; however, the authors were able to demonstrate that the AAF adducts in the DNA were not deacetylated to AF adducts when the shuttle vector was replicated *in vivo*.

The mutagenesis results observed by Maher and coworkers correlate with the earlier findings of Carothers et al. (1988) and Moriya et al. (1988); however, the mutational data for AAF observed in studies conducted in mammalian cells do not agree with the results observed in bacterial cells (Koffel-Schwartz et al., 1984). Differences in the mutagenic processing of AAF and AF adducts between bacterial and mammalian cells or the effect of unknown adduct repair mechanisms in mammalian cells may account for the differences observed in these two systems.

Aromatic Amines and Oncogene Activation

The accurate repair of carcinogen-DNA adducts in the endogenous genes of mammalian cells is crucial. Mutations caused either during repair or replication of carcinogen-adducted DNA can be important if they cause DNA sequence changes that result in the activation of proto-oncogenes (Topal, 1988). Activating mutations in oncogenes have been suggested to initiate neoplastic changes in the phenotype of the normal cell to a pre-cancerous type (Weinberg, 1989).

Two early studies investigated activated oncogenes in cell lines derived from human bladder tumors (Tabin et al., 1982; Reddy et al., 1982).

These studies demonstrated that activated oncogenes were found in both the EJ and T24 human bladder carcinoma cell lines; in both cases the genetic change that led to the activation was a G -> T point mutation at the second position of the 12th codon, GGC, of the oncogene (Tabin et al., 1982; Reddy et al., 1982). Characterization of the oncogene revealed that it was a member of the H-*ras* family, oncogenes found in many types of tumors (Fujita et al., 1984).

One of the first studies that many workers have interpreted to show that chemical carcinogens can activate oncogenes *in vivo* was conducted by Zarbl et al. (1985). The results of this study demonstrated that mammary tumors induced in rats by the chemical carcinogen, *N*-methyl-*N*-nitrosourea, show a G -> A mutation at the second position of the 12th codon, GGA, of the H-*ras* gene. While it has not been directly shown that an O^6-alkyl DNA adduct caused the mammary tumor formation (other models are possible), it is noteworthy that the type of mutation induced by O^6-methylguanine *in vivo* (Loechler et al., 1984; Ellison et al., 1989) matches that observed in mammary tumors by Zarbl and coworkers.

The discovery of the H-*ras* oncogenes combined with the knowledge that activation of these genes can occur by single bp mutations has been viewed to lend support to the somatic mutation theory of cancer and the important role of chemical carcinogens in the process of tumorigenesis (Brookes, 1989). It is emphasized, however, that other models for tumorigenesis by chemical carcinogens are possible (R. Cha, unpublished observations).

In addition to the G -> T mutations found at the second position of the 12th codon, GGC, of the H-*ras* gene in bladder tumors, A -> T mutations at

the 61st codon, CAG, have been reported (Nishimura and Sekiya, 1987; Lawley, 1990). G -> C and G -> T changes at the first position of 12th codon, GGT, of the K-*ras* gene have also been observed in bladder tumor cells.

Studies investigating the mechanism of aromatic amine-induced carcinogenesis have taken two approaches to identifying activating oncogene mutations. In the first approach, Vousden and colleagues have transfected a plasmid containing the human H-*ras* gene treated *in vitro* with AAAF into NIH 3T3 cells (Vousden et al., 1986). The activating oncogene mutations found were primarily C:G -> A:T and G:C -> T:A transversions at the first and third positions, respectively, of the 61st codon, CAG, of the *ras* gene. Another study investigated AAF-induced tumors in mice (Wiseman et al., 1986). These investigators treated animals with N-OH-AAF and analyzed the activating mutations by the NIH 3T3 assay. Of 7 AAF-induced hepatomas, 7 were found to contain a C:G -> A:T mutation at the first position of the 61st codon, CAA, of the H-*ras* gene.

A recent study demonstrated that BZ cogeners can activate *ras* oncogenes in rats treated with these compounds (Reynolds et al., 1990). The majority of the activating mutations that occurred in the *ras* gene represented three groups: G:C -> A:T mutations at the first and second positions of the 12th codon, GGA; G:C -> C:G mutations at the first position of codon 13, GGC, and C:G -> A:T mutations at the first position of the 61st codon, CAA, of the H-*ras* gene.

In addition to the involvement of *ras* oncogenes in carcinogenesis, the presence of mutated or deleted forms of tumor suppressors genes such as the

p53 gene in human cancers has been documented (Vogelstein, 1990). A recent study has demonstrated mutations in the *p53* gene in 61% of bladder tumors that were analyzed (Sidransky et al., 1991). The types of base changes that could have led to the alterations in the *p53* gene include G -> C, G -> A, and G -> T mutations.

The results of these studies suggest a good correlation between the types of mutational changes induced by chemical carcinogens observed in mutagenesis studies and the changes that have been observed to activate proto-oncogenes or inactivate tumor suppressor genes *in vivo*. These studies validate the use of *in vitro* assays to determine the mutagenic potential as well as the mutational specificity of a chemical carcinogen.

III. MATERIALS AND METHODS

Chemicals

4-Nitrobiphenyl, acetic anhydride and trifluoroacetic anhydride were purchased from Aldrich Chemical Co. *N*-Hydroxy-aminobiphenyl (2,2'-[^{3}H], 92 mCi/mmol) was purchased from Midwest Research Institute. Reagents for synthesis of deoxyoligonucleotides were obtained from Applied Biosystems. HPLC solvents were from Omnisolv. Adenosine triphosphate (ATP), Sepharose CL-4B chromatography resin and ethidium bromide were purchased from Sigma Chemical Co. DNA-grade hydroxylapatite (HAP), 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) and isopropyl β-D-thiogalactopyranoside (IPTG) were purchased from Boehringer Mannheim Biochemicals. Radionuclides [γ^{32}P]-ATP (6000 Ci/mmole) and [α^{35}S]-ATP (600 Ci/mmole) were obtained from Dupont/New England Nuclear and Amersham Corporation, respectively. Acrylamide, Bio-Gel A agarose resin, and polyacrylamide resin P4 were from Bio-Rad Laboratories.

DNA Standards and Primers

The 8-32 base oligonucleotide molecular weight markers were purchased from Pharmacia LKB Biotechnology. The lambda *Hind*III digest DNA markers were purchased from Gibco Bethesda Research Laboratories (BRL). The M13 *lac*Z gene 17-base sequencing primer was synthesized at the Whitehead Institute for Biomedical Sciences (Cambridge, MA). The M13 cloning region 15-base primer was purchased from New England Biolabs. The M13 "-40"

17-base sequencing primer was purchased either from Pharmacia LKB Biotechnology or the United States Biochemical Corporation. The 15-base oligonucleotides used for the hybridization analysis were synthesized in the Biopolymers Laboratory (MIT). DNA sequencing reagents were purchased from either Amersham, Pharmacia LKB Biotechnology or the United States Biochemical Corporation.

Enzymes

Exonuclease III (ExoIII) was purchased from Gibco BRL. DNA restriction enzymes *Pst*I, *Sma*I, and *Bgl*II were purchased from Boehringer-Mannheim Biochemicals or New England Biolabs. *E. coli* DNA polymerase I (Klenow fragment), calf intestinal alkaline phosphatase (CIP), bacteriophage T4 polynucleotide kinase (T4 PNK), and T4 DNA ligase were purchased from Boehringer Mannheim Biochemicals. Bacteriophage T4 polynucleotide kinase was also obtained from New England Biolabs. T7 DNA polymerase, *E. coli* DNA ligase and nuclease P_1 were purchased from Pharmacia LKB Biotechnology. Sequenase T7 DNA polymerase enzyme version 2.0 was obtained from the United States Biochemical Corporation. Snake venom phosphodiesterase (type II) was purchased from Sigma Chemical Co.

Bacterial Strains

The cell strains used in this study were derivatives of the *E. coli* AB1157 (*lac*$^+$, *uvr*$^+$) strain, constructed in our laboratory (Lasko et al. 1988), and are listed as follows: excision repair-proficient *E. coli* DL7 cells ((AB1157, lac ΔU169, *uvr*$^+$); and the isogenic excision-repair deficient *E. coli* DL6 cells (AB1886, lac ΔU169, *uvrA*) (Table 1).

Table 1.

Bacterial Strains

E. coli strain	Relevant genotype	Source
DL7	AB11157 ; *lac* Δ*U169* ; *uvr*$^+$	D. Lasko
DL6	AB1886 ; *lac* Δ*U169* ; *uvrA*	D. Lasko
DL5	AB1885; *lac* Δ*U169* ; *uvrB*	D. Lasko
DL4	AB1884 ; *lac* Δ*U169* ; *uvrC*	D. Lasko
DL7/pGW16	AB1157 ; *lac* Δ*U169* ; *uvr*$^+$; (pGW16: *mucA*; *mucB*)	D. Lasko
MM294A	*lac*$^+$	K. Backman
GW5100	JM103 P1$^-$; *lacZ* ΔM15 ; M13 plating strain; *amber* suppressor (*supE*)	L. Marsh
NR8044	*lacZ* ΔM15 ; M13 plating strain; *opal* suppressor	R. Dunn

In addition, DL7 and DL6 cells induced for SOS functions by UV-irradiation (Defais et al., 1976; Lasko et al., 1988) and DL7 cells containing the mutagenesis enhancing plasmid, pGW16 (a derivative of pKM101; Walker, 1978), were used for transfection of ABP-modified M13 genomes.

Bacteriophage Vectors

The bacteriophage M13mp10 stock used for producing the M13 single-stranded and replicative form DNAs for generating the randomly ABP-modified genomes was purchased from Pharmacia LKB Biotechnology. The initial bacteriophage stocks of M13mp19 insert genomes, M13mp19(+6) and M13mp19(+10), used to make the gapped heteroduplexes vectors for the site-specific mutagenesis studies were a gift from M. Benasutti and E. Loechler (Boston University, Boston, MA) (see Figure 8).

Bacterial Culture Media

Media supplies for cell growth were from Difco laboratories. Luria-Bertani (LB) media (1% tryptone, 0.5% yeast extract, 1.0% NaCl; Maniatis, 1982) was used to grow *E. coli* DL7, DL6 and DL7/pGW16 cells for transformation by M13 ss and RF DNAs. *E. coli* DL7 and derivatives were propagated on LB plates containing 1.5% Bacto-agar. SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose; Hanahan, 1985) was used for *E. coli* cells after the electrotransformation protocol. The M13 host cells were propagated on M13 minimal A (Miller, 1972) plates containing 0.01% thiamine. Single colonies of M13 host plating strains were grown in 2X YT media (1.6% tryptone, 1.0%

yeast extract, 1.0% NaCl; Messing, 1983). M13 bacteriophage-infected or M13 DNA-transfected cells were plated in B Broth soft agar (1.0% tryptone, 0.8% NaCl, 0.6% Bacto-agar; Messing, 1983) containing X-gal (0.5 $\mu g/\mu L$) and IPTG (0.2 $\mu g/\mu L$) with fresh *E. coli* M13 host cells on B broth indicator plates (1.0% tryptone, 0.8% NaCl, 2.0% Bacto-agar).

Preparation of M13 Bacteriophage DNA

Large-Scale Isolation of M13 RF and SS DNA

M13 bacteriophage ss DNA was prepared by a modified protocol of Messing (1983). For large-scale isolation of M13 ss and RF DNAs, one liter (L) cultures of an M13 plating bacteria strain (approximately 10^6 cells/mL) were inoculated with an amplified M13 bacteriophage stock (approx. 10^{10} PFU/mL) and grown overnight in 2X YT medium at 37 °C. The following day the bacterial/phage culture was spun in 250-ml centrifuge bottles in a Sorvall centrifuge to pellet the bacterial cells. The pelleted cells were saved to isolate the M13 RF DNA. M13 ss DNA was harvested from the supernatant by PEG 8000/NaCl (20% PEG, 2.5M NaCl) precipitation. The precipitated phage was resuspended in TE (10 mM Tris-Cl, 1.0 mM EDTA pH 8.0), extracted with phenol buffered with 0.1 M Tris-Cl (pH 8.0) (1X), phenol-chloroform (1:1) (3X), chloroform (1X) and finally the phage ss DNA was precipitated with ethanol and resuspended in TE. The ss DNA was further purified over a HAP column equilibrated in phosphate buffer (0.14 M KH_2PO_4; 0.14 M K_2HPO_4) and stored in TE at 4 °C.

M13 RF DNA was isolated from the phage-infected bacterial cells using a modified version of the Triton-lysis procedure (Lasko et al., 1986;

Sambrook et al., 1989). Briefly, the cell pellet from a 1 L phage/bacterial culture was resuspended in Sucrose-Tris-saline buffer, and lysed in a 0.5% Triton-Tris-EDTA-SDS solution. The lower molecular weight nucleic acids were purified from the chromosomal DNA and the cell debris, which were pelleted in an ultracentrifugation spin. The closed circular RF DNA was further separated from nicked DNA and RNA by centrifugation overnight in cesium chloride-ethidium bromide gradient conditions. The RF DNA was removed from the gradient tubes, extracted with butanol to remove the ethidium bromide, subsequently dialyzed against 1X TE to remove the CsCl, and stored in TE at 4 °C.

Small-scale Isolation of M13 RF and SS DNA

For analysis of M13 mutant phage samples, small-scale preparations of M13 ss and RF DNA were made. Briefly, 1.5 mL of 2X YT was inoculated 1:100 with an late-log phase culture of *E. coli* GW5100 and a single M13 phage plaque and grown in a culture tube for 3 hours (hr) on a tube rollerdrum at 37 °C. The cells were pelleted in 1.5 mL microfuge tubes and saved to isolate M13 RF DNA as described below. M13 ss DNA was isolated from the bacteriophage in the bacterial culture supernatant by a modified protocol of Messing (1983). Briefly, the phage supernatant was respun in a microfuge to pellet any remaining cells and added to a tube containing 1/5 volume 20% PEG, 2.5 M NaOAc (pH 7.0) and incubated at 4 °C. Following this incubation, the solution was centrifuged to precipitate the phage; subsequently, the PEG solution was removed and the phage pellet was resuspended in TE. The sample was extracted 1X with phenol, 1X with chloroform and precipitated with 1/10 volume 3 M NaOAc (pH 5.2) and 2.5

volumes of ethanol. After incubation at -70 °C, the DNA was precipitated by centrifugation, the pellet was washed 2X with 70% ethanol, dried and resuspended in TE.

M13 RF DNA was isolated from phage-infected cells according to a modified alkaline lysis protocol (Sambrook et al., 1989). The cell pellet was resuspended in 10 mM Tris-EDTA, 100 mM NaCl buffer and recentrifuged to remove any residual supernatant. The cells were again resuspended in 50 mM glucose, 25 mM Tris-Cl, and 10 mM EDTA (pH 8.0) and placed on ice. A 0.2 N NaOH, 1.0% SDS lysis solution was added to the cell suspension and the tubes were gently mixed and placed on ice until the solution was clear. Subsequently, a 5 M potassium acetate solution was added to the tubes, the samples were mixed well, and kept on ice. Following the incubation on ice, the tubes were centrifuged to pellet the cell debris and the large chromosomal DNA. The supernatant was removed and extracted 1X with phenol:chloroform:isoamyl alcohol (25:24:1) and the aqueous layer was precipitated with 2.5 volumes of ethanol at 25 °C. The samples were centrifuged at 4 °C, and washed with 70% ethanol. The DNA was dried and resuspended in 100 μL of TE. The RNA was precipitated from the solution by adding a equal volume of 10 M lithium chloride, incubating the tubes on ice, followed by centrifugation to pellet the large RNAs. The supernatant was precipitated with 2.5 volumes of ethanol and precipitated at -20 °C. The DNA/ethanol solution was centrifuged to isolate the DNA; the pellet was washed with 70% ethanol, dried and resuspended in TE.

Mutagenesis and Toxicity of Ultraviolet Light to M13 Genomes

The aim of the UV mutagenesis assays was to determine the optimal amount of UV required for induction of mutagenic SOS functions in both excision repair proficient and excision repair deficient bacterial strains. To achieve this goal, UV survival assays were conducted first, by direct irradiation of *E. coli* cells and determining cell survival and second, by transfecting irradiated M13 DNA into cells that were UV irradiated or not irradiated for induction of SOS functions.

Genotoxicity of UV irradiation in E. coli

For UV survival studies, *E. coli* strains DL7, DL7/pGW16, and DL4 cells were irradiated for various times with UV irradiation from a 254 nm light source. UV fluence rate was measured with a UVX radiometer; typical rates were 1.0-1.2 Joules/meter^2·second (J/m^2·s). Briefly, 100 mL of sterile LB media was inoculated with 1 mL of an overnight culture of *E. coli* DL7, DL7/pGW16 or DL4 cells and grown in 500-mL baffled culture flasks until mid-log phase, usually 1-2 x 10^8 cells/mL. Cells were then placed on ice and harvested in 250 mL Sorvall bottles (Oak-Ridge type) by spinning at 5000*g* (7000 rpm) in a Sorvall RC2B centrifuge. The supernatant was discarded and the cell pellet was resuspended in 100 mL of sterile, cold 10 mM $MgSO_4$. At this step, all but one-fifth of the cells was removed for UV irradiation for induction of host SOS gene functions. The *uvr*$^+$ cells, DL7 and DL7/pGW16, were exposed in 25 ml aliquots in sterile 150 mm petri dishes to a range of UV doses from 8-50 seconds (s) to a 15 W General Electric germicidal lamp at a UV fluence rate of 1.1 J/m^2·s. DL4 (*uvrC*)

cells were resuspended in cold LB media and irradiated as above to a range of UV doses from 2-12 secs. Aliquots of cells were removed and plated on LB plates to determine cell survival before and after different amounts of UV irradiation.

Mutagenesis Induced by UV Lesions in M13 DNA

To assess the genotoxic and mutagenic effects of UV lesions in *E. coli*, M13mp10 or M13mp18 bacteriophage ss DNAs were UV irradiated and then transfected into competent *E. coli* cells that were UV irradiated or not irradiated to induce SOS functions. After irradiation of the bacterial cells, both the UV-treated and control cells were added in separate flasks to an equal volume of 2X LB and incubated at 37 °C for 40 minutes (min), during which time expression of SOS gene products occurred (Defais et al., 1976). The *uvrC* cells were added to an equal volume of LB and incubated at 37 °C for 20 min. After the SOS incubation period, the cells were placed on ice and harvested by centrifugation for 10 min at 5000*g*. The cells were then made competent for uptake of DNA by the calcium chloride method (Mandel and Higa, 1970; Cohen et al., 1972; Maniatis, 1982). Briefly, the cell pellets were resuspended in an equal volume of sterile, cold 30 mM $CaCl_2$ and placed on ice for 20 min. The cell suspension was again centrifuged at 5000*g* for 5 min and the pellet was resuspended in 1/20 volume of cold 30 mM $CaCl_2$, placed on ice and used as soon as possible for the transfection of modified DNAs. Typically, for each transfection, 2 μg of M13 ss DNA was irradiated for 60 s with a 254 nm lamp at a UV fluence of 1.1 $J/m^2 \cdot s$ and mixed with 300 μL of *E. coli* calcium chloride-competent cells. For control transfections, 0.1 μg of M13 ss DNA was transfected

into 300 μL competent bacteria. Transfection samples were then held on ice (0 °C) for 15-30 min, heat-shocked at 42 °C for 90 s and subsequently plated with M13 host cells on B Broth indicator plates, containing X-gal and IPTG. Mutant phage, detected as light blue and colorless among wild-type blue plaques, were enumerated to determine the mutation frequency.

These UV mutation experiments were conducted first, to determine if the M13 bacteriophage ss genome was an adequate vector in which to study mutagenesis; and second, to assess if the UV irradiation conditions were sufficient to induce the SOS mutagenic processing functions of the host cells.

Mutagenesis Assays with Globally ABP-Modified M13 Genomes

ABP mutagenesis assays in the *lacZ* gene of randomly ABP-modified M13 DNA were performed to determine which of the known DNA adducts of ABP were likely to be mutagenic in the M13 ss genome and to compare the mutational spectrum in ss DNA with the results observed in our laboratory with ABP-modified ds DNA (Lasko et al., 1988). Cells of different repair backgrounds were used for the mutation assays with globally modified DNAs to investigate which bacterial genes are important in the repair or mutagenesis of ABP-DNA lesions.

ABP Modification of M13 Genomes

Modification of M13 ss and RF DNAs was conducted with activated *N*-OAc-TFAABP, in the laboratory of F. Kadlubar (NCTR). *N*-OAc-TFAABP was synthesized from *N*-hydroxy-ABP (2,2'-[^{3}H]) by the method of Lee and King

(1981). M13mp10 ss DNAs (7244 bases) were randomly adducted with 20 mM *N*-OAc-TFAABP (specific activity 7.85 mCi/mmole). Reaction conditions were 300 μg/mL M13 ss DNA in TE (pH 8.0) at 37 °C for 1 hr. Amounts of *N*-OAc-TFAABP to add to the reaction were calculated to achieve levels of modification of 1 to 20 lesions per genome. M13mp10 RF DNAs (7244 bp) were modified in a similar reaction at a concentration of 200 μg/ml to achieve levels of 10 to 100 adducts per duplex genome. DNA buffer controls (b.c.) using ethanol instead of ABP were used to assess the effect of reaction conditions on the DNA. Two hundred to three hundred μg of ds and ss DNA were used and a total of three dose points including a b.c. was done for each modification experiment. The DNA samples were purified by n-butanol and phenol extractions to remove unreacted *N*-OAc-TFAABP and the modified DNAs were stored in ethanol at -70 °C. As a standard, calf thymus DNA modified with *N*-OAc-TFAABP under similar conditions, was hydrolyzed and analyzed by HPLC to determine the distribution of adducts and the adduct profile of DNA binding by this activated ABP compound.

The extent of modification (the number of [^{3}H]-ABP residues bound to DNA) was determined by measuring the amount of radioactive material present in a known amount of DNA and calculating the number of adducts present. The number of apurinic (AP) sites possibly caused by the reaction conditions was assessed by the method of Drinkwater et al. (1980) and as described in Lasko et al. (1988). Modified RF DNA was treated with ExoIII under conditions where it acts as an AP endonuclease (in the presence of Ca^{2+}). The percent of conversion of supercoiled RF DNA (form I) to nicked circular RF DNA (form II) or linear RF DNA (form III) was determined using

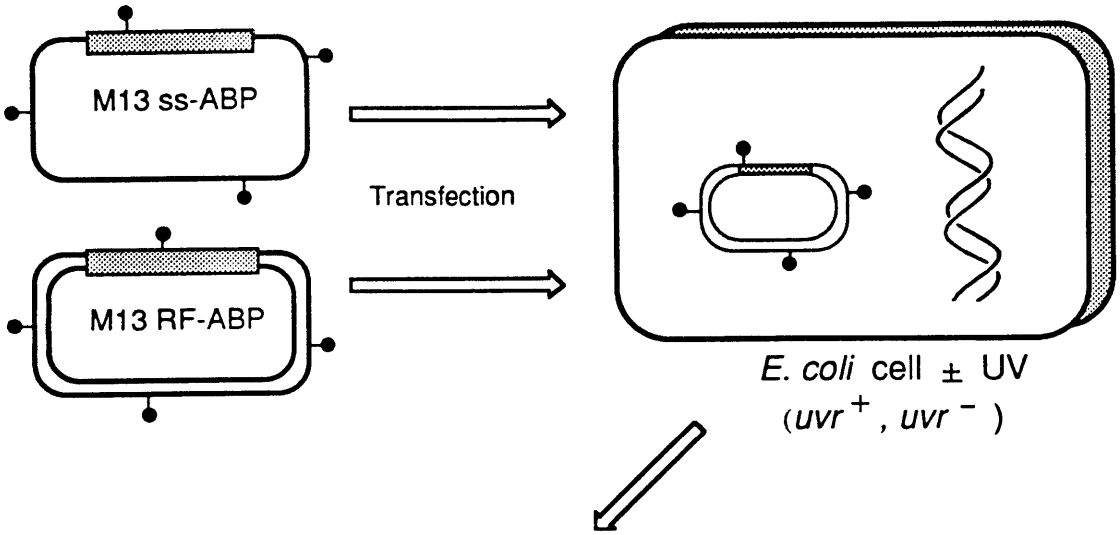
positive/negative film and densitometric evaluation. The mean number of strand breaks (m) is estimated from the relation $m = -\ln f_1$ where f_1 is the amount of the DNA that remains as Form I after treatment with Exo III. The number of AP sites was estimated in this manner. M13 ABP-modified ss DNA was treated with alkali, 0.1 N NaOH, and electrophoresed in 1.0% agarose gels (1X TBE) stained with ethidium bromide to assess the integrity of the M13 ss DNA and estimate the number of putative AP sites that may have formed after the ABP reaction conditions.

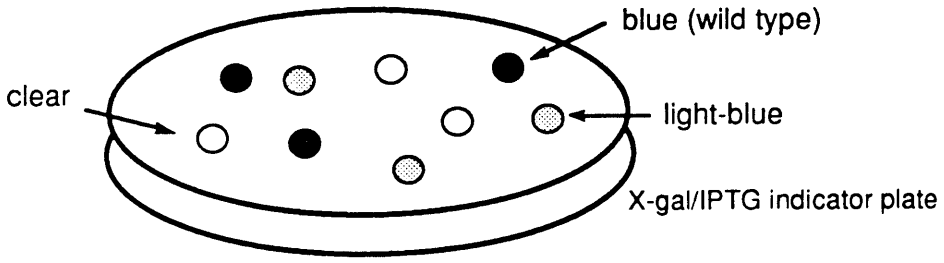
Transfection of Globally ABP-modified M13 DNAs into E. coli

M13 ss and RF DNAs randomly modified with ABP were transfected into *E. coli* cells where they were subjected to the repair and replication systems of the host (Figure 10). The *E. coli* cells used for the transfection were made competent for uptake of exogenous DNA by the calcium chloride protocol as follows. Typically, 100 mL of sterile LB media was inoculated with 1 mL of an overnight culture of *E. coli* DL7, DL7/pGW16 or DL6 cells and grown in 500-mL culture flasks until mid-log phase, usually 1-2 x 10^8 cells/mL. Cells were then placed on ice and harvested in 250 mL Sorvall bottles by spinning at 5000*g* in a Sorvall centrifuge. The supernatant was discarded and the cell pellet was resuspended in 100 mL of sterile, cold 10 mM $MgSO_4$. At this step, half of the cells was removed for UV irradiation in order to induce the host SOS gene functions. The *uvr*$^+$ cells, DL7 and DL7/pGW16, were exposed for 40 secs to a 15 W General Electric germicidal lamp at a UV fluence rate of 1.1 J/m^2·s in 25 ml aliquots in sterile 150 mm petri dishes. After irradiation, both the UV-treated and control cells were added in separate flasks to an equal volume of 2X LB and incubated at 37 °C

Figure 10.

Mutagenesis Assays in the *lac*Z gene of bacteriophage M13. ABP-modified M13 single- and double-stranded DNAs were transfected into competent *E. coli*. Transfectants were plated on indicator plates with M13 host bacterial cells. Mutants were scored as light-blue or colorless plaques among blue wild-type plaques. Mutation Frequency was determined by dividing the mutant plaques by the total number of plaques.





Score for colorless mutants or light-blue mutants

Mutation frequency :

$$\frac{\text{\# light-blue or colorless plaques}}{\text{Total \# of plaques (LB + C + B)}}$$

Sequence analysis of mutants

Mutational spectrum of ABP in *E. coli*

for 40 min for expression of SOS gene products. DL6 (*uvrA*) cells were resuspended in cold LB media and irradiated as above for 6 secs and added to an equal volume of LB and incubated at 37 °C for 20 min. An aliquot of the cells was removed before and after UV treatment to compare cell survival before and after UV exposure. After the SOS incubation period, the cells were placed on ice and harvested by centrifugation for 10 min at 5000*g*. The cells were made competent by the calcium chloride method as described above. The competent cells were placed on ice and used as soon as possible for the transfection of modified DNAs.

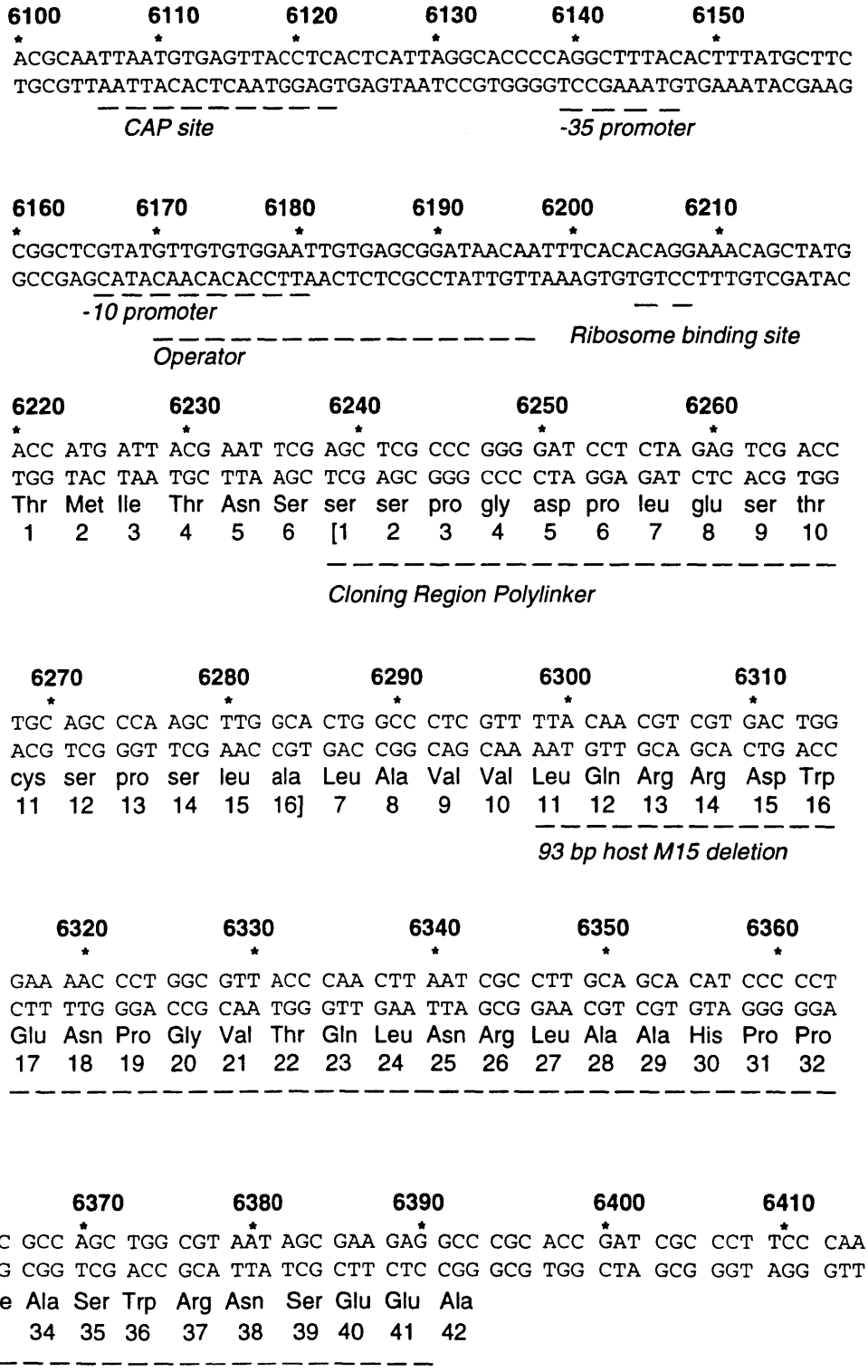
M13 RF and ss DNAs were transfected into competent DL7, DL7/pGW16, or DL6 cells. For ABP-modified ss DNA, 50 ng of the b.c. DNA, 100-750 ng of modified ss DNA or 1 ng of control ss DNA in a total volume of 100 μL and approximately 200 μL (4.0×10^8) of cells was used for each transfection. Forty ng of RF-modified DNA and 1 ng of b.c. DNA were used per transfection. The cell-DNA mixture was incubated in culture tubes on ice for 30-60 min and then heat-shocked at 42 °C for 90 s. Aliquots of the transfection mixture were then plated with host bacteria (*E. coli* GW5100) on IPTG, X-gal indicator B broth plates. Transfection efficiency or survival of the modified genomes was determined by enumerating infective centers produced after plating the transfection mixture and comparing this number to the actual amount of DNA used per transfection.

Mutant Isolation and DNA Sequencing Methods

Mutations in the *lacZα* gene can be detected as colorless or pale-blue plaques compared to blue (wild-type) plaques. The mutation frequency was determined by dividing the number of mutant phage by the total number of infective centers obtained after the transfection. Putative mutant phage were plaque-purified by restreaking on new plates and small-scale preparations of M13 ss DNA were obtained for sequence analysis. M13 ss DNAs were sequenced by the dideoxy method (Sanger et al., 1977) to analyze the types of mutations induced by ABP-DNA lesions. A synthesized 15-base primer (Kunkel, 1984) that anneals to position 6407-6422 of the M13mp10 genome was used to sequence the 300 bp *lacZα* target in the M13 genome (Figure 11). Sequencing reactions were performed with a reagent kit and [α^{35}S]-ATP according to the protocol provided by the manufacturer. The reactions were electrophoresed in 6% or 8% polyacrylamide (19:1 acrylamide:bisacrylamide), 1X TBE gels containing 7M urea (Maniatis et al., 1982). After electrophoresis, gels were fixed in 10% methanol, 10% glacial acetic acid and dried at 80 °C on a gel dryer. Exposures to Kodak X-Ray film were carried out for 16-24 hours at room temperature. The sequencing data was used to formulate a mutation spectrum for ABP in the *lacZ* gene of M13.

Figure 11.

The *lacZα* gene target of M13mp10. The cloning region polylinker and the 93 bp deletion present in the host bacteria (*lacZ* ΔM15) are denoted (adapted from Lasko et al., 1988).


6100 6110 6120 6130 6140 6150
ACGCAATTAATGTGAGTTACCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTC
TGCGTTAATTACACTCAATGGAGTGAGTAATCCGTGGGGTCCGAAATGTGAAATACGAAG
CAP site
-35 promoter
6160 6170 6180 6190 6200 6210
CGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATG
GCCGAGCATACAACACACCTTAACTCTCGCCTATTGTTAAAGTGTGTCCTTTGTCGATAC
-10 promoter
Operator
Ribosome binding site
6220 6230 6240 6250 6260
ACC ATG ATT ACG AAT TCG AGC TCG CCC GGG GAT CCT CTA GAG TCG ACC
TGG TAC TAA TGC TTA AGC TCG AGC GGG CCC CTA GGA GAT CTC ACG TGG
Thr Met Ile Thr Asn Ser ser ser pro gly asp pro leu glu ser thr
1 2 3 4 5 6 [1 2 3 4 5 6 7 8 9 10
Cloning Region Polylinker
6270 6280 6290 6300 6310
TGC AGC CCA AGC TTG GCA CTG GCC CTC GTT TTA CAA CGT CGT GAC TGG
ACG TCG GGT TCG AAC CGT GAC CGG CAG CAA AAT GTT GCA GCA CTG ACC
cys ser pro ser leu ala Leu Ala Val Val Leu Gln Arg Arg Asp Trp
11 12 13 14 15 16] 7 8 9 10 11 12 13 14 15 16
93 bp host M15 deletion
6320 6330 6340 6350 6360
GAA AAC CCT GGC GTT ACC CAA CTT AAT CGC CTT GCA GCA CAT CCC CCT
CTT TTG GGA CCG CAA TGG GTT GAA TTA GCG GAA CGT CGT GTA GGG GGA
Glu Asn Pro Gly Val Thr Gln Leu Asn Arg Leu Ala Ala His Pro Pro
17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
6370 6380 6390 6400 6410
TTC GCC AGC TGG CGT AAT AGC GAA GAG GCC CGC ACC GAT CGC CCT TCC CAA CAG
AAG CGG TCG ACC GCA TTA TCG CTT CTC CGG GCG TGG CTA GCG GGT AGG GTT GTC
Phe Ala Ser Trp Arg Asn Ser Glu Glu Ala
33 34 35 36 37 38 39 40 41 42

Site-Specific Mutagenesis Assays with Singly ABP-Modified M13 Genomes

Subsequent to obtaining the mutational spectrum for ABP in M13 ss DNA, the next step was to determine the mutagenic specificity of the major deoxyguanosine adduct of ABP, dG-C8-ABP, in M13 ss DNA. This experiment would establish the contribution of this lesion to the entire mutational spectrum. In order to do this, I have constructed a site-specifically modified M13 genome containing a single dG-C8-ABP lesion. This genome was made by ligating either an ABP-adducted tetranucleotide, 5'-d[pTpG-(ABP)pCpA]-3', or the corresponding unmodified tetramer, 5'-d[pTpGpCpA]-3', both of which were synthesized in our laboratory, into a unique *Pst*I restriction enzyme site in a gapped heteroduplex M13 vector. The details of the synthesis and characterization of TG-(ABP)CA (MIT), the construction of site-specifically ABP-modified genomes using the tetranucleotide, TG-(ABP)CA, the transfection of modified genomes into *E. coli* cells, and the mutant selection protocols are described in the following sections.

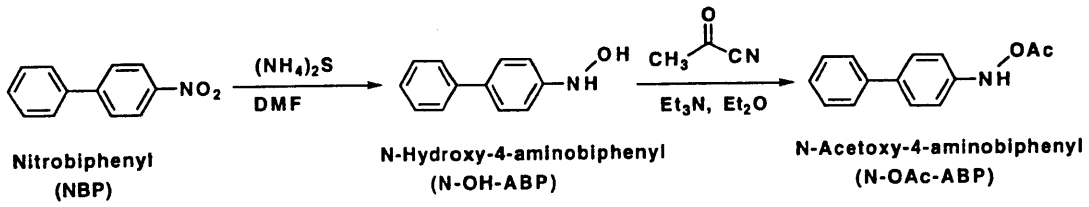
Synthesis, Purification and Characterization of a Tetranucleotide Containing the Major DNA Adduct of ABP

Synthesis of the deoxytetranucleotide 5'-d[TpG-(ABP)pCpA]-3'

The deoxytetranucleotide, 5'-d[TpGpCpA]-3', synthesized by the cyanoethyl phosphoramidite method, was modified with *N*-acetoxy-4-aminobiphenyl (*N*-OAc-ABP), an electrophilic derivative, to yield ABP-adducted d[TGCA] (Figure 12). The *N*-OAc-ABP used in the synthesis of

Figure 12.

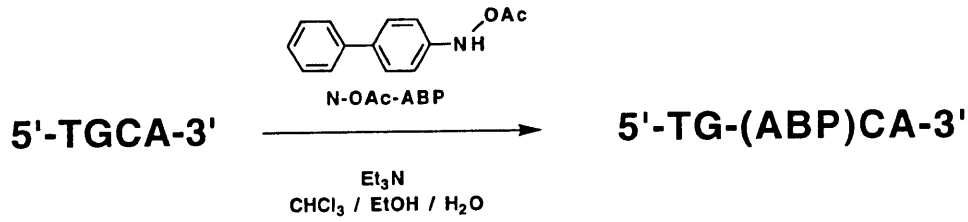
Synthesis of the Tetranucleotide 5'-pTpG-(ABP)pCpA-3' (MIT). The tetranucleotide 5'-TGCA-3' was reacted with N-acetoxy-4-aminobiphenyl to yield the ABP-adducted tetranucleotide, 5'-TG-(ABP)CA-3.'. Details are provided in the text.

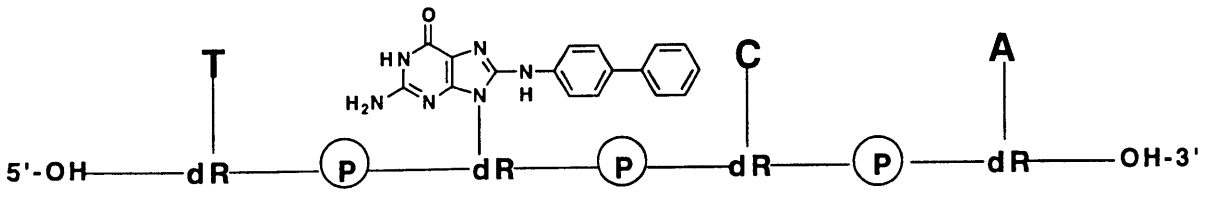


Nitrobiphenyl (NBP)

N-Hydroxy-4-aminobiphenyl (N-OH-ABP)

N-Acetoxy-4-aminobiphenyl (N-OAc-ABP)





5'-TpG-(ABP)pCpA-3'

5'-d[TpG-(ABP)pCpA]-3' was prepared by reduction of 4-nitrobiphenyl (*N*O-ABP) to *N*-OH-ABP and subsequently *N*-acetylated in a reaction with pyruvonitrile and triethylamine that was incubated at 45 °C for 1 hr. A 10 μmol portion of TGCA dissolved in a mixture of chloroform : ethyl alcohol : H_2O and triethylamine was allowed to react with five sequential additions of solid *N*-OAc-ABP (total 3.78 mmol) at 25 °C. After 3 hr, the reaction was diluted with dichloromethane and water, the aqueous layer was collected and washed with dichloromethane and finally the sample was concentrated to dryness *in vacuo*.

Purification and Characterization of 5'-d[TpG-(ABP)pCpA]-3'

The crude mixture from the reaction of TGCA with N-OAc-ABP was purified by a preparative reversed phase HPLC system equipped with a Hewlett Packard 1040A diode-array detector using a linear gradient from 0-50% acetonitrile in 0.1 M ammonium acetate pH 5.8. The tetranucleotides d[TGCA] and d[TG-(ABP)CA] were further analyzed on a Phenomenex Ultramex 5-μm C_{18} column (25 x .46 cm) using a reversed phase HPLC Beckman system. After HPLC purification the tetramers were desalted using the same HPLC column and a linear H_2O, CH_3CN gradient. The tetramers were characterized during HPLC purification by UV spectroscopy at 260 nm and 310 nm (to detect aromatic amine-substituted DNA molecules) using the diode array detector to confirm the structural integrity of the ABP-adducted tetramer.

Nucleotide Composition Analysis

After the tetranucleotides were purified, both the modified and unmodified tetramers were enzymatically digested to their respective nucleosides, which were then analyzed by HPLC to confirm the nucleoside composition. Digestion was performed sequentially with nuclease P_1 for 12 hr at 37 °C in 10 mM $ZnSO_4$, 30 mM NaOAc (pH 5.2) then snake venom phosphodiesterase for 3-4 hr at 37 °C in 100 mM Tris-Cl (pH 8.0), 100 mM $MgCl_2$. The products of the digestion were analyzed on a C_{18} reversed phase column using a gradient from 0-50% CH_3CN in 0.1 M NH_4OAc pH 7.2.

Electrophoretic Gel Analysis of Tetranucleotides

In conjunction with the HPLC purification, both the unmodified and ABP-modified oligonucleotides were 5'-end labeled with [γ^{32}P]-ATP to yield (5'-^{32}P)-d[TpGpCpA]-3' and (5'-^{32}P)-d[TpG-(ABP)pCpA]-3', respectively. The tetranucleotides were then denatured at 100 °C and electrophoresed on 23% polyacrylamide gels to assess the purity and integrity of the samples. In separate reactions the ABP-adducted and control tetranucleotides were subjected to the same conditions used to denature the modified and unmodified genomes. After treatment at 100 °C for 5 min, the oligonucleotides were analyzed by UV spectroscopy and HPLC to assess the chemical stability of the tetramers under these conditions.

After characterization of 5'-TG-(ABP)CA-3' and 5'-TGCA-3', the 5'-phosphorylated tetranucleotides were ligated into M13 gapped duplex vectors to produce the site-specifically modified and unmodified genomes, respectively.

Construction of a Site-specifically Modified M13 Genome Containing the Major dG-C8-ABP Adduct at a Unique *Pst*I Restriction Enzyme Site

Preparation of M13 Gapped Heteroduplex Vectors

A site-specifically ABP-modified M13 genome was constructed by ligating a 5'-phosphorylated ABP-adducted tetranucleotide into a four-base gap in a gapped heteroduplex vector (M13GHD) that had a nick in the strand opposite that containing the gap. Using the same protocol a control genome was constructed with the unmodified tetranucleotide. The M13 gapped duplexes were made from a pair of M13mp19 derived vectors according to a modification of the protocol of Green et al. (1984). In a typical reaction, 50 μg of an M13mp19 genome denoted as M13mp19(+6) was linearized by *Sma*I restriction enzyme digestion and annealed to 50 μg of a genome, M13mp19(+10), containing a unique *Pst*I site that had been linearized at a unique *Bgl*II and 5'-dephosphorylated with calf alkaline intestinal phosphatase (CIP). The gapped duplex reaction was first incubated at 100 °C for 5 min, then 65 °C for 30 min and then slowly cooled to room temperature. After the reaction, an aliquot of the mixture was run on a 0.8% agarose gel (stained with ethidium bromide to visualize the DNA) to determine the percent of formation of M13GHD. This method could two produce two forms of the gapped heteroduplexes, vectors containing a unique 4-base gap at the *Pst*I site in the plus strand of the M13 duplex and a nick at the *Bgl*II site in the minus strand or vectors containing the 4-base gap in the minus strand of the M13 genome and the nick present in the plus strand of the viral genome. The phosphorylated tetranucleotides could be

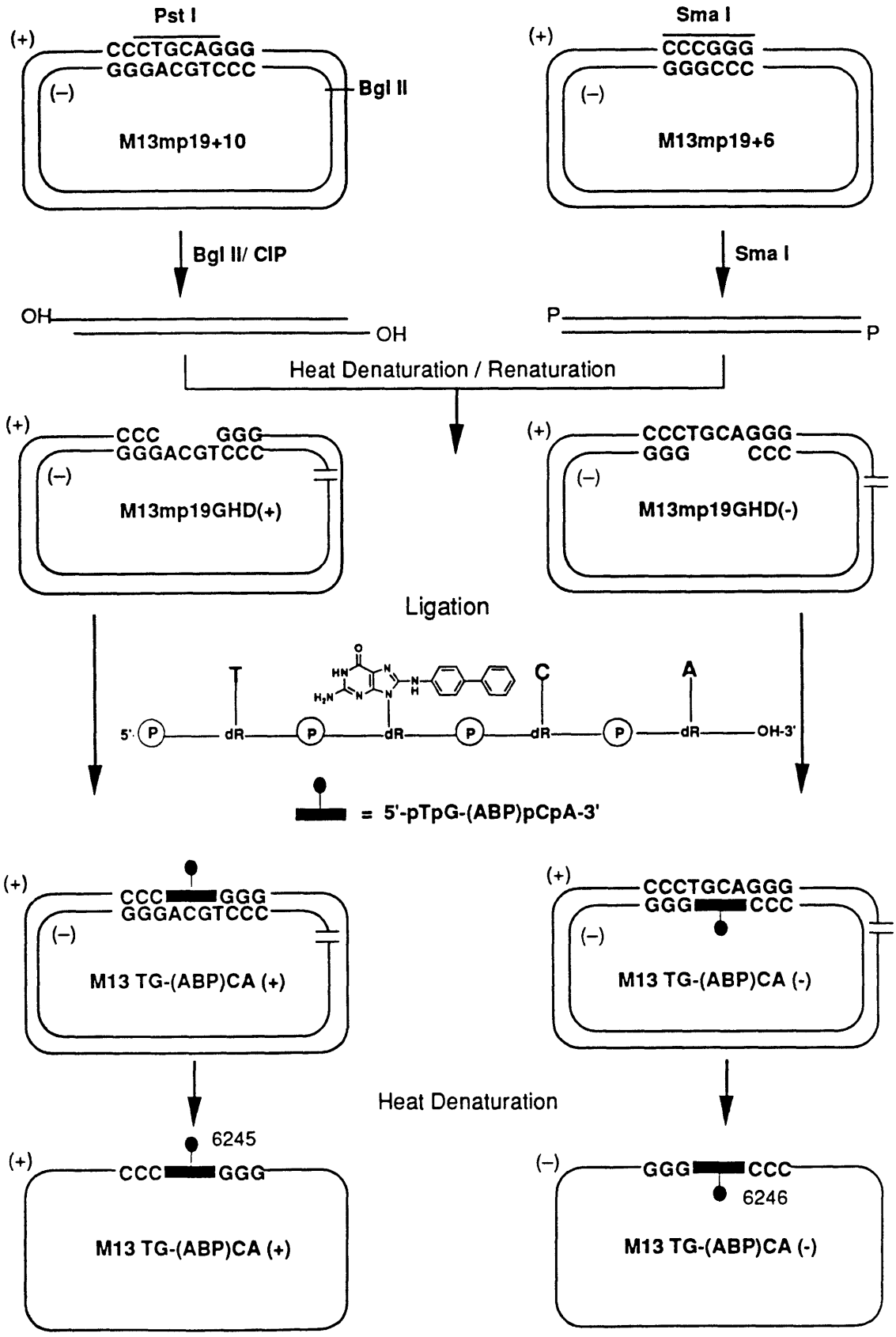
ligated into the four-base gap in either form of the gapped duplex vectors (Figure 13). The M13GHD vectors were made fresh and stored in aqueous solution at 4 °C until the ligation experiment.

Phosphorylation and Characterization of 5'-TpG-(ABP)pCpA-3' and 5'-TpGpCpA-3'

The tetranucleotides used in the construction of the singly-adducted genomes were 5'-phosphorylated and then ligated into the gapped heteroduplex vectors. For the pilot experiments, typically, 100 pmoles (133 ng) of the ABP-modified tetranucleotide, 5'-d[TpG-(ABP)pCpA]-3' or 100 pmoles (118 ng) of the unmodified tetramer, 5'-d[TpGpCpA]-3' were 5'-phosphorylated with 100 μCi of [γ^{32}P]-ATP (6000 Ci/mmol) using T4 polynucleotide kinase in 50 mM Tris-Cl, 10 mM $MgCl_2$, 5 mM DTT (pH 7.4) and unlabeled ATP at a five-fold molar excess in a 10 μL reaction. Samples were incubated at 37 °C for 45 min after which the reaction was terminated at 65 °C for 15 min. Following the reaction, an aliquot of the phosphorylation reaction, usually one pmole, was analyzed on a 23%, 7 M urea, 1X TBE denaturing polyacrylamide gel. The radiolabeled tetramers were visualized as bands on the gel by autoradiography of the wet gel to X-ray film or exposure to a solid phosphor screen. Analysis of the phosphor screen was carried out on a Molecular Dynamics phosphorimager. Using the autoradiograph as a marker, the labeled tetramer bands were sliced from the gel and counted (Cerenkov) in a Beckman scintillation counter. In this manner, the specific activity of the tetranucleotide could be determined as cpm per pmole of oligonucleotide.

Figure 13.

Construction of a site-specifically modified genome containing a single N-(deoxyguanosin-8-yl)-4-aminobiphenyl adduct at a unique *Pst*I site in an M13mp19+ genome. M13mp19(+10) RF DNA was linearized at a unique *Bgl*II site and 5'-dephosphorylated with calf alkaline intestinal phosphatase (CIP). This M13-*Bgl*II/CIP RF DNA was heat denatured and renatured with M13mp19(+6) RF DNA that was digested with *Sma*I to produce two forms of the M13mp19 gapped heteroduplex vectors (M13GHD). A 5'phosphorylated dG-C8-ABP-modified tetranucleotide, 5'-pTpG-(ABP)pCpA-3', was ligated into a four-base gap of a unique *Pst*I restriction site of the M13GHD. M13 genomes containing the dG-C8-ABP adduct in either the plus strand (+) at position 6245, or the minus (-) strand at 6246 were produced from the ligation with a nick at the *Bgl*II site in the strand opposite that containing the adduct. In parallel, a control genome was constructed with an unmodified tetranucleotide, 5'-pTpGpCpA-3'. Subsequently, the modified and the unmodified genomes could be heat denatured to produce the singly ABP-adducted ss genome, M13-TG-(ABP)CA, and the control ss genome, M13-TGCA.


Pst I
(+)
CCCTGCAGGG
GGGACGTCCC
(–)
Bgl II
M13mp19+10
Sma I
(+)
CCCGGG
GGGCCC
(–)
M13mp19+6
Bgl II/ CIP
Sma I
OH
OH
P
P
Heat Denaturation / Renaturation
(+)
CCC GGG
GGGACGTCCC
(–)
M13mp19GHD(+)
(+)
CCCTGCAGGG
GGG CCC
(–)
M13mp19GHD(-)
Ligation
T
C
A
5'
P
dR
OH-3'
= 5'-pTpG-(ABP)pCpA-3'
(+)
CCC GGG
GGGACGTCCC
(–)
M13 TG-(ABP)CA (+)
(+)
CCCTGCAGGG
GGG CCC
(–)
M13 TG-(ABP)CA (-)
Heat Denaturation
6245
(+)
CCC GGG
M13 TG-(ABP)CA (+)
(–)
GGG CCC
6246
M13 TG-(ABP)CA (-)

Ligation of Tetranucleotides into Gapped Heteroduplex Vectors

Subsequently, in parallel reactions, the ABP-adducted and the control [^{32}P]-phosphorylated tetramers were ligated at a five-hundred-fold molar excess into 0.2 pmole (1 μg) of the gapped duplex genome. Ligation reactions were performed with either *E. coli* DNA ligase for 16 hr at 12°C in 20 mM Tris-Cl (pH 7.5), 4 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.1 M KCl containing 0.1 mM NAD^+ or Bacteriophage T4 DNA ligase for 16 hr at 16°C in 50 mM Tris-Cl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, and 1 mM ATP). Following the ligation reaction, the ligated genomes were purified from unincorporated tetramers by chromatography through a 0.7 cm x 20 cm column containing Sepaharose CL 4B or BioRad Bio-Gel A desalting gel. In some instances, prior to column chromatography, the ligation mixture was spun in a Centricon-10 microconcentrator (Amicon Corporation) to remove the excess of unincorporated radiolabeled tetramer. The presence of the [γ-^{32}P]-ATP label was used to monitor the ligated genomes during purification by column chromatography and subsequent gel analysis.

Characterization of Site-specifically ABP-modified M13 Genomes

The efficiency of ligation of the ABP tetramer, 5'-pTpG-(ABP)pCpA-3' into the gapped duplexes was determined by calculating the specific activity of the site-specifically modified genomes in cpm per pmol of genome. The ligation efficiency represents the percent of M13GHD that had incorporated a phosphorylated ABP-tetramer. This value is compared to the ligation efficiency of the control tetramer, 5'-pTpGpCpA-3', into the gapped duplex vector.

The ABP-modified genome was characterized with restriction endonuclease digests to confirm the location of the adduct in the genome as well as to observe if the presence of the adduct inhibited *Pst*I restriction enzyme cleavage at the *Pst*I site. The unmodified genome was digested in parallel as a control reaction. Restriction digests and uncut samples were then analyzed in 0.8% agarose gels containing ethidium bromide. After electrophoresis, the gels were photographed and then dried at 60 °C on a gel dryer and exposed to X-ray film overnight with an intensifying screen or exposed to a phosphor screen to visualize the radiolabeled genomes.

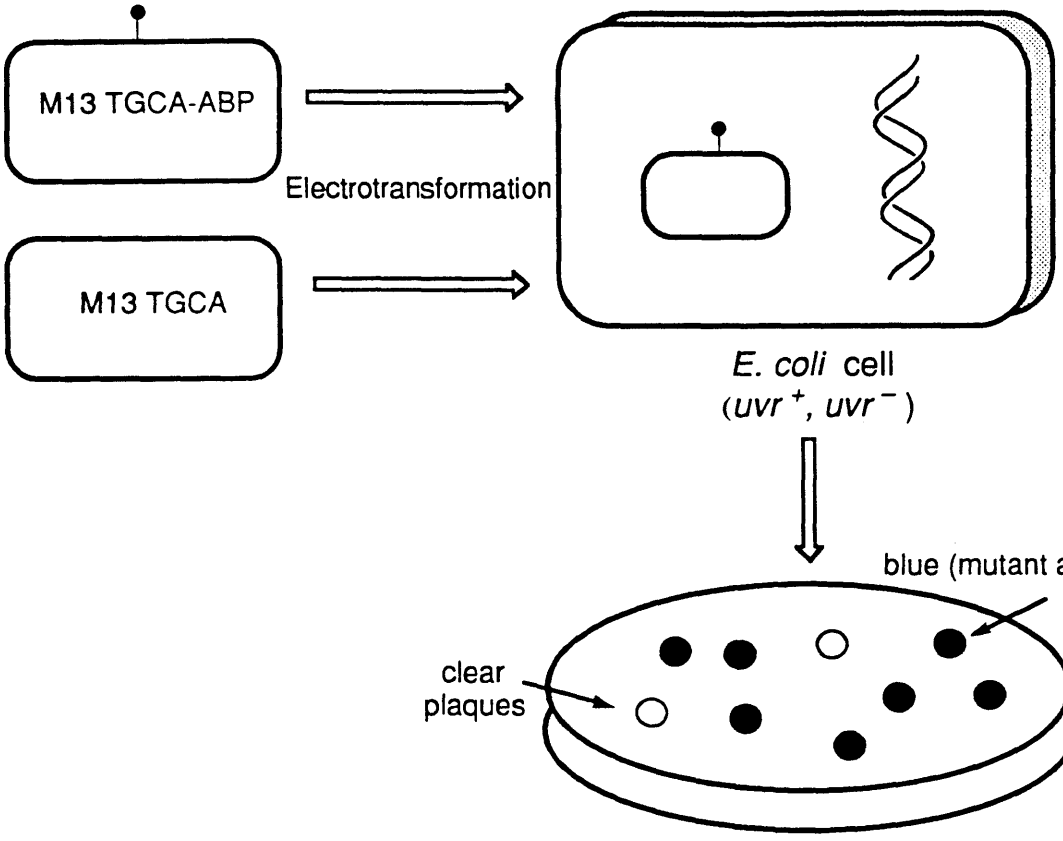
In addition, the radiolabeled, nicked, duplex M13 genomes were denatured with heat (100 °C for 5 min) and electrophoresed in 1.0% agarose gels that were stained with ethidium bromide following gel electrophoresis. This analysis was used to determine the actual percentage of the ligation sample that represented viable ss DNA.

Transfection Assays with a Singly-ABP-adducted M13 Genome in *E. coli*

The site-specifically ABP-modified genomes and control genomes used for the mutagenesis experiments were constructed prior to each experiment as described above. Mutagenesis experiments involved replicating both the control bacteriophage genome, M13-TGCA, and the adducted genome, M13-TG-(ABP)CA, into *E. coli* DL7 (*uvr*$^+$) cells or *E. coli* DL7/pGW16 (*uvr*$^+$/*mucAB*) cells that were induced for SOS functions by UV-irradiation (Figure 14). The transfectants were plated on an *E. coli* M13 host strain and the progeny phage were analyzed to determine the genotoxic and mutagenic effects of a single ABP lesion in this cell system. In parallel

Figure 14.

Site-specific mutagenesis assays with the ABP-modifed single-stranded genome, M13TG-(ABP)CA, and the control genome, M13TGCA. Genomes were transfected by electroporation into cells that were induced or not induced for SOS functions. Transfectants were plated to determine transfection efficiency of the site-specifically modified genome. Progeny phage were plated, mutants were isolated and sequenced to determine the mutation frequency as well as the mutation specificity of the dG-C8-ABP adduct.



Mutation frequency:

$$\left(\frac{\text{\# Mutant blue phage}}{\text{\# Total blue phage}}\right) X \left(\begin{matrix}\text{internal standard} = \\ \text{\# clear phage}\end{matrix}\right)$$

Selection for mutants

Sequence analysis of mutants

Mutation specificity of dG-C8-ABP in *E. coli*

experiments, UV irradiated *E. coli* DL7/pGW16 cells were transfected with UV-irradiated M13mp18 ss DNA to assess if the UV irradiation conditions were adequate for induction of SOS functions.

Preparation of and UV Irradiation of Cells

The cells used for these mutagenesis experiments were grown, UV treated and prepared for DNA transfection as follows. One mL of an overnight culture of *E. coli* DL7 cells or *E. coli* DL7/pGW16 cells was diluted into 100 mL of LB medium and grown separately in 500-ml baffled flasks until in mid-log phase, usually 1-2 x 10^8 cells/ml. The cells were placed on ice for 5 min and then spun in 250 ml Sorvall bottles at 5000*g* (7000 rpm, 10 min) in a Sorvall centrifuge to pellet the cells. After the supernatant was removed the cells were resuspended in an equal volume of sterile, cold 10 mM $MgSO_4$.

At this step, the DL7 cells were kept on ice and the DL7/pGW16 cells were removed to be UV-irradiated for induction of SOS gene functions. DL7/pGW16 cells were exposed for 40 s to a 15 W General Electric germicidal lamp at a UV fluence rate of 1.1 $J/m^2 \cdot s$ in 25 ml aliquots in sterile 150 mm petri dishes. An aliquot of the cells was removed before and after UV treatment to compare cell survival before and after UV exposure. After irradiation, both the UV-treated and control cells were added in separate flasks to an equal volume of 2X LB and incubated at 37 °C for 40 min for expression of SOS gene products. After the recovery, both flasks of cells were placed on ice and prepared and washed with H_2O to remove particulates

that can interfere with the electrotransformation protocol that was used for transfection of M13 DNA (Dower et al., 1988).

Briefly, 200 mL of cells were spun in two 250 mL Sorvall bottles as described above. The supernatant was removed and the cells were resuspended in a total of 100 mL of sterile, ice-cold H_2O, pooled and spun at 5000*g* (7000 rpm, 15 min). The H_2O was removed carefully and the cell pellet was then resuspended in 25 mL of sterile H_2O and transferred to a 50 mL sterile Sorvall tube and spun at 5000*g* (7000 rpm, 15 min). The H_2O was removed from the tubes with a sterile 25 mL pipet and the cell pellet was finally resuspended with H_2O (if required) in a total volume of 200 μL and placed on ice to be used as soon as possible.

Preparation of M13 Genomes for Transfection

While growing the cells, the control and adducted genomes were prepared for the transfection. Both sets of duplex vectors were digested with *Sma*I and only the ABP-modified genomes were treated with *Pst*I to ensure that any *Pst*I sensitive material was removed from the sample. The digested samples were extracted with phenol:chloroform 1X, chloroform 1X, then purified and desalted with 3 washes of TE in a Centricon-10 microconcentrator. Prior to each transfection, the duplex genomes were heat denatured at 100 °C for 5 min to generate ss genomes and then placed on ice to prevent strand renaturation.

Transfection of M13 Genomes into E. coli Cells by Electroporation

For each electrotransformation, 40 μL of cells were added to microfuge tubes containing denatured genomes and this mixture was transferred to a

cold Bio-Rad Gene Pulser cuvette. Cells were electroporated with a Bio-Rad Gene Pulser electroporation system at 2.5 kV volts, 25 μF and 200 ohms with an average time constant of 4.7 s (Dower et al., 1988). To assess the effect of the electroporation conditions on the cells, aliquots of the cells were plated before and after electroporation to determine cell survival. Immediately after electroporation, one ml of SOC medium was added to the transformation mixture and the samples were removed from the cuvette to a sterile culture tube. Aliquots of the mixture were plated on B broth, X-Gal, IPTG indicator plates with an M13 host *E. coli* GW5100 to determine efficiency of transfection of the genomes. Survival of the modified genomes was measured by enumerating infective centers that were produced after plating an aliquot of the transfection. Progeny phage were produced by incubating the transfection tube at 37°C for 2 hr. After the incubation, the sample was centrifuged to pellet the cells and aliquots of the progeny phage from the supernatant were diluted in TE, plated and enumerated. Progeny phage from the initial transfection were subsequently analyzed for mutants using the selection protocols detailed in the following section.

Isolation and Selection of DNA Mutants

PstI Restriction Enzyme Selection

Several methods were used to isolate and enumerate mutant phage among the original progeny phage. The first protocol that was used to select for mutants was similar to that of Loechler et al. (1984), for a site-specifically-modified genome containing O^6-methylguanine. Since the ABP adduct resides in a unique *Pst*I restriction site, 5'-CTGCAG-3', in the polylinker cloning region in the *lac*Z gene of the M13 genome, any missense mutation (except one change, *vide infra*) at this site does not affect the production of active β-galactosidase and hence there is no phenotypic difference in the blue plaque color exhibited by the wild-type (wt) phage compared to the mutant phage in this assay. Any mutation that does occur at the *Pst*I site, however, does inactivate the recognition site for the *Pst*I restriction enzyme and this was the rationale used to select for mutant phage among the wild-type population.

After the transfection experiment, progeny phage were produced and the M13 RF DNA was isolated from the phage-infected cells. Typically 5×10^5 phage were incubated with 2×10^7 cells in 5 mL of 2X YT medium and grown in a culture tube at 37 °C for 4-5 hr. The RF DNA was isolated from the phage-infected cells as described earlier. The RF DNA was first subjected to restriction by *Pst*I enzyme (25 units for 10 μg RF DNA) to linearize wild-type RF DNA, and subsequently transformed into *E. coli* MM294A cells made competent by the $CaCl_2$ method.

This mutant selection protocol was based upon two assumptions: first, the adduct in the *Pst*I site rendered mutant progeny resistant to this enzyme, and second, linear RF DNA transforms at a very low efficiency (< 1.0% compared to circular RF DNA). Rounds of mutant selection were continued to amplify mutant phage with respect to wt phage until there was no apparent further increase in the relative number of *Pst*I resistant phage. After the final transformation, putative mutant phage were selected for sequence analysis at the *Pst*I site. Small-scale preparations of RF DNA were done as described above, 5-10 μL of the sample was then removed and digested with *Pst*I enzyme. If the sample was resistant to *Pst*I cleavage, phage DNA was isolated from the phage culture supernatant and sequenced to determine the types of mutations induced at the *Pst*I site. The mutation frequency was determined by enumerating the number of blue mutant progeny phage from the final transformation and dividing that by the total number of phage from the initial transformation and using a colorless plaque phenotype as an internal standard to correct for transformation efficiency.

In-Frame opal Mutation

One type of expected base substitution mutation was readily screened for in the *lacZα* mutation assay. This is a G:C > T:A bp substitution in the minus strand at position 6246 that will result in a TGA (*opal* stop) codon in the reading frame of the β-galactosidase gene in the *lac*Z gene of the M13 genome. These mutations can be detected as colorless plaques against wild-type blue plaques if the phage are plated on an M13 plating host *E. coli* GW5100, an *amber* suppressor strain. If, however, the same colorless plaques are picked and plated on an *opal* suppressor strain, *E.*

coli NR8044, the TGA stop codon will be suppressed by the host cell and the mutant plaques will have a pale-blue phenotype. M13 phage DNA can be isolated from these putative mutants and the mutation can be confirmed by sequence analysis.

M13 Plaque Hybridization Protocol

A differential plaque hybridization technique was used as an alternative method of mutant isolation to confirm the results obtained from the *Pst*I rounds selection method. After transfection, phage plaques produced on a plate were transferred by stippling in a grid onto a new plate that had an overlay of the *opal* suppressor host, *E. coli* strain NR8044, grown for 2 hours. In a typical experiment, 10 plates each with approximately 300 stippled plaques would be analyzed for each sample. The plaques from the grid were lifted by overlaying a 137 mm dry nitrocellulose filter (Schleicher and Schuell) onto the plate. After the filters were air-dried, they were baked for 30 min on a vacuum gel dryer at 80 °C.

The filters were then screened with a 15-base 5′-[^{32}P]-end-labeled oligonucleotide, 5′-CGCCCTGCAGGGCAA-3′, that had a complementary sequence to the wild-type phage DNA at the *Pst*I restriction site. In a typical reaction, 200 pmoles of the 15-mer was 5′-end-labeled with 500 μCi of [γ^{32}P]ATP and 30 units of T4 polynucleotide kinase. The hybridization conditions used for the plaque-lift assay were 6X SSPE, 10% dextran sulfate, 0.5% sodium pyrophosphate, 0.1% SDS, 100 μg/mL sonicated calf thymus DNA with a probe concentration of 8 ng/mL at 1 x 10^6 cpm/mL. Single filters were placed in 150 mm petri dishes with 25 mL of hybridization solution and incubated at 42 °C for 16-24 hr with slight agitation to

decrease nonspecific binding. The filters were then washed in large Pyrex crystallizing dishes with four changes of 200 mL of 6X SSPE, 0.1% SDS solution equilibrated at 42 °C. After the final wash, the filters were placed on a sheet of 3 MM paper which was covered in Saran wrap and exposed under slight pressure to a phosphor screen for 24-72 hr. The phosphor screen was scanned and hybridization analysis was performed on the Molecular Dynamics phosphorimager. Using this method of hybridization analysis, the putative mutant plaques were detected, and subsequently plaque-purified and analyzed by DNA sequencing.

Characterization of Mutant DNAs

Putative mutant phage isolated from the mutant selection protocols were plaque-purified by restreaking onto new plates and small-scale preparations of single plaques were made. The purified M13 ss DNA was sequenced using the dideoxynucleotide sequencing method of Sanger et al. (1977). Alternatively, M13 RF DNA was isolated from phage-infected cells produced from a single putative mutant plaque and restricted with *Pst*I enzyme and analyzed by gel electrophoresis. M13 ss DNAs from the samples that were resistant to enzyme digestion were prepared for sequence analysis.

DNA sequence analysis of mutants

Sequencing reagents using [^{35}S-ATP] and Sequenase T7 DNA Polymerase enzyme were used to sequence the putative mutant DNAs. The primer used was the "-40" M13 17-base sequencing primer that anneals at position 6278-6294, just outside the polylinker region in the vector (the adduct was positioned in the *Pst*I site, at position 6245 or 6246 in the polylinker) (Figure 8).

Sequences were analyzed on 10% acrylamide (19:1 acrylamide:bis acrylamide), 1X TBE, 7M urea sequencing gels, that were fixed, dried and exposed for 24 hrs to X-Ray film.

IV. RESULTS

Genotoxicity and Mutagenicity of UV Light in *E. coli*

The UV toxicity and mutagenesis assays were conducted to determine the optimal amount of UV required for induction of mutagenic SOS functions in both excision repair proficient (uvr^+) and excision repair deficient (uvr^-) bacterial strains. UV survival assays were carried out by direct UV irradiation of *E. coli* cells and determining cell survival. The UV genotoxicity and mutagenicity assays were performed by transfecting UV-irradiated M13 DNA into cells that were UV-treated or not treated for induction of SOS functions.

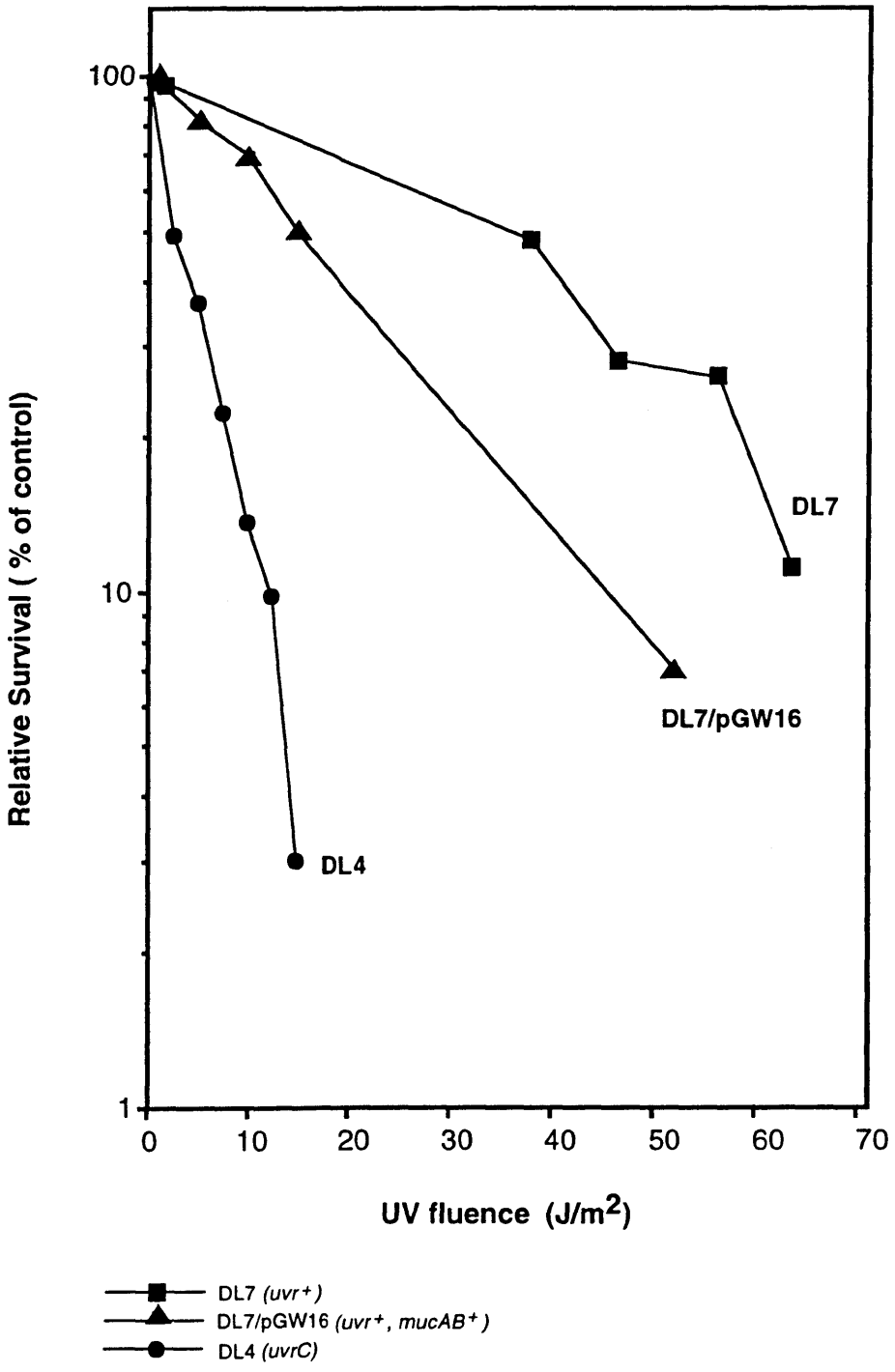
UV Toxicity Assays

The *E. coli* uvr^+ strains, DL7 and DL7/pGW16 (pGW16: *mucAB*), and the *uvrC* strain, DL4, were irradiated with increasing doses of UV light to determine cell survival after UV irradiation. The results of the UV cell survival assays are shown in Figure 15. Two observations were made based on these results: first, the uvr^+ strains, DL7 and DL7/pGW16, were more resistant to the toxic effects of UV irradiation than the uvr^- DL4 cells; and second, cells harboring the plasmid pGW16, DL7/pGW16, were more sensitive to UV irradiation than DL7 cells without the plasmid.

The toxic effect of UV was clearly observed in the *uvrA* cells, which were more sensitive to UV radiation at much lower doses than the uvr^+ strains. The 37% survival, corresponding to one lethal hit, was attained at approximately 45 J/m^2, 20 J/m^2 and 5 J/m^2 for the uvr^+, uvr^+/*mucAB* and

Figure 15.

Genotoxicity of Ultraviolet Light in *E. coli*. UV survival assays were performed by UV-irradiating *E. coli* with a range of UV doses and plating aliquots of irradiated and unirradiated cells to determine cell survival.


100
10
1
0
10
20
30
40
50
60
70
Relative Survival (% of control)
UV fluence (J/m2)
DL7
DL7/pGW16
DL4
DL7 (uvr+)
DL7/pGW16 (uvr+, mucAB+)
DL4 (uvrC)

uvrA cells, respectively. The survival results confirm the involvement of the *uvr* excision repair system on the survival of the cell after cellular DNA damage by UV irradiation.

UV Mutagenesis Assays with UV-Irradiated M13 Genomes

These assays involved transfecting UV-irradiated M13 ss genomes into *E. coli* cells irradiated with increasing doses of UV light. The results were used to determine the optimum UV radiation dosage required to induce the SOS mutagenic functions of the host cells. Either M13mp10 or M13mp18 ss DNA UV irradiated at 72 J/m^2 was transfected in parallel with unirradiated M13mp10 or M13mp18 ss DNA into *E. coli* cells made competent for DNA uptake by the calcium chloride method. Transfectants were plated on indicator plates and mutations in the *lacZ* gene represented as light-blue or colorless plaques among wild-type blue plaques were enumerated. The mutation frequency was determined by dividing the number of mutant plaques by the total number of infective centers.

The results of the UV mutagenesis assays indicate a significant increase in mutation frequency of UV-irradiated DNA in UV-irradiated host cells compared to unirradiated cells (Table 2). At equivalent doses of UV treatment to DL7 and DL7/pGW16 cells, there was a three-fold increase in mutation frequency in UV-DNA replicated in cells containing pGW16 compared to cells without the plasmid. Approximately equal mutation frequencies were obtained for UV-DNA in both the uvr^+ and the *uvrA* strains. The results suggest that the amount of UV required to induce SOS functions at a cell survival level of 10-40% was 45 J/m^2 and 7 J/m^2 for the uvr^+ and *uvrA* strains, respectively.

Table 2.

Mutagenesis by UV-irradiated M13 ss DNA MF = (x 10^{-4})[a]

Cells		UV dose to cells (J/m^2): 0	37	45	55
	DNA UV dose (J/m^2)				
DL7 (*uvr*⁺)	**0**	2.9	2.5	<7.9	<6.6
	60	n.d.	6.4	36	8.6

Cells		UV dose to cells (J/m^2): 0	9.6	50
	DNA UV dose (J/m^2)			
DL7/pGW16 (*uvr*⁺/*mucAB*)	**0**	8.4	10	17
	60	44	46	102

Cells		UV dose to cells (J/m^2): 0	4.8	7.2	9.6
	DNA UV dose (J/m^2)				
DL4 (uvrA)	**0**	n.d.	2.5	<2.5	<2.5
	60	<3.1	4.7	28	6.1

[a] MF= x 10^{-4} . Mutation frequency is the number of mutant plaques divided by the total number of plaques (infective centers)

n.d. not determined

The Mutational Spectrum of ABP in the M13 SS Genome

The first step towards generating a mutational spectrum for ABP in the *lacZ* gene of M13 was to conduct forward mutation assays in *E. coli* with globally ABP-modified bacteriophage genomes. The assay used to evaluate the mutagenicity of ABP-DNA adducts in the *lacZ* gene detects many bp substitutions as well as most insertions and deletions (Kunkel, 1984).

Analysis of ABP-Modified M13 Genomes

M13 ss and RF DNAs were adducted to various adduct levels with *N*-OAc-TFAABP. Modification of DNA by this compound produces a similar adduct profile to reaction of DNA with *N*-OH-ABP (Beland and Kadlubar, 1985; Lasko et al., 1988). *N*-OAc-TFAABP reacted with M13 ss DNA more efficiently than ds DNA; therefore, much less ABP reagent was required to achieve the same number of adducts for ss DNA as for ds DNA. The adduct levels obtained from the reaction were 0 (b.c.), 1 and 5 lesions per 7 kilobase (kb) ss genome and 0 (b.c.), 45 and 93 adducts per 7 kbp RF genome. When the ABP-modified genomes were characterized by gel electrophoresis, the M13 ss and RF DNAs migrated on the gel with ss and RF DNA standards, suggesting that the reaction conditions did not obviously affect the genomes. When the AP site analysis was conducted, the RF genomes containing 93 ABP adducts were estimated to contain less than 1 AP site per genome. Analysis of ABP-modified ss genomes indicated that there were no significant differences between alkali-treated b.c. DNA and ABP-modified DNA.

Transfection of ABP-Modified DNAs into E. coli

The bacterial strains used for the transfection of M13 ABP-modified DNAs were the *E. coli* strains, DL7 (*uvr*$^+$), DL6 (*uvrA*), DL7/pGW16 (*uvr*$^+$/*mucAB*) and DL6/pGW16 (*uvrA/mucAB*), induced or not induced for SOS functions by UV irradiation (Table 1). The modified DNAs were transfected into cells made competent by the $CaCl_2$ method. Subsequently, transfectants were plated on indicator medium plates and infective centers produced from the transfection were enumerated for determining transfection efficiency and mutation frequency (Figure 10). Mutant phage, detected as light-blue or colorless plaques, were distinguished from the blue (wild-type) phage on indicator plates as described above.

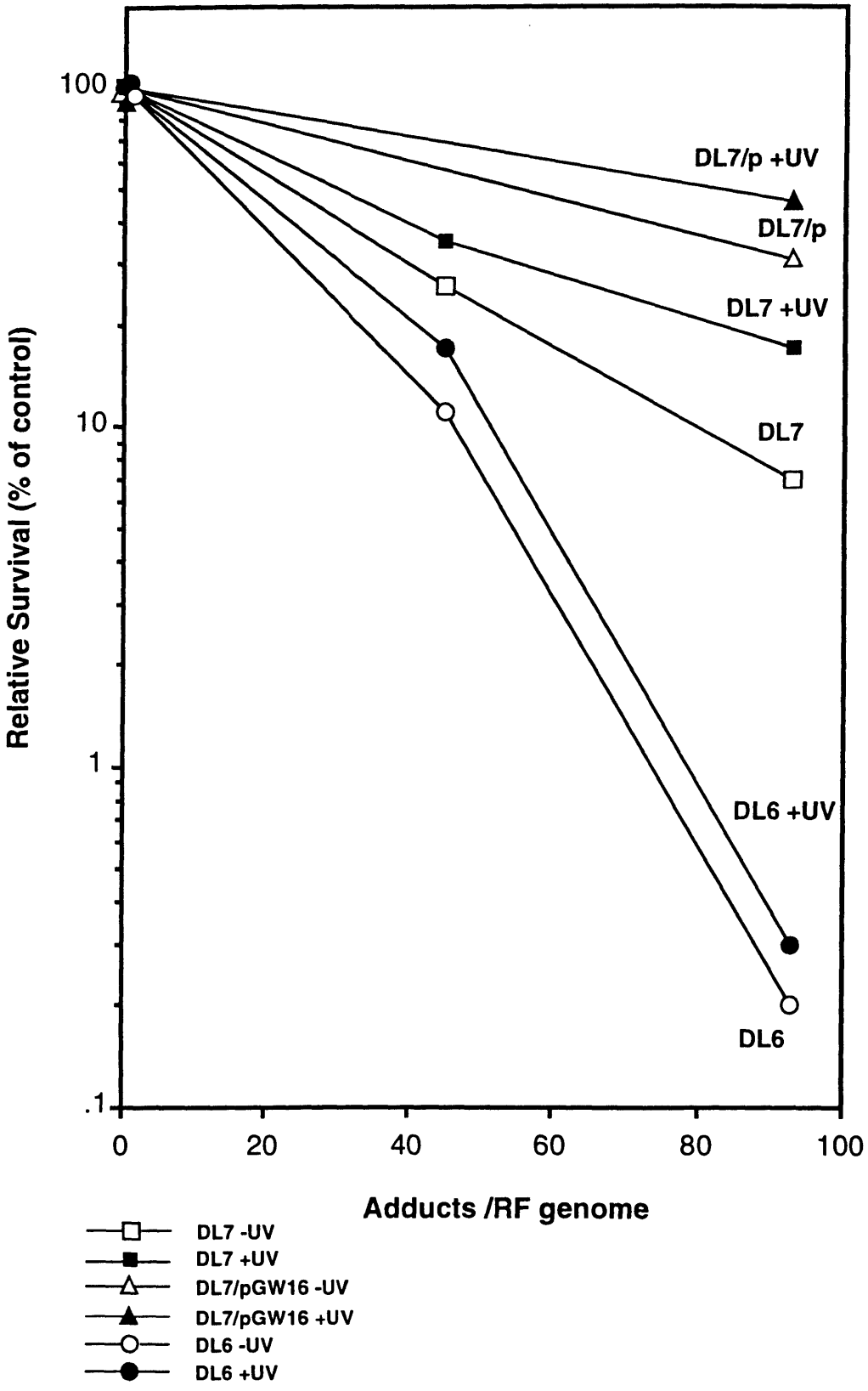
Genotoxicity of ABP-DNA Lesions in M13 Genomes

Survival of M13 RF ABP-Modified DNA

The results indicate that survival of ABP-modified M13 RF genomes decreased with increasing adduct levels; however, survival of ABP-adducted ds DNA, especially at 93 adducts/genome, was enhanced by the excision repair-proficiency of the host cell (Figure 16). In *uvr*$^+$ DL7 cells induced for SOS mutagenic processing, the survival of ds DNA (93 adducts/genome) was two-fold higher than in cells not UV-irradiated and increased to 6-fold higher in DL7 cells containing the plasmid pGW16. The transfection efficiency of genomes containing 93 ABP lesions was much lower in DL6 *uvrA* cells and this number was not significantly increased in UV-irradiated host cells. The genotoxicity of ABP lesions was more profound in *uvrA* cells

Figure 16.

Genotoxicity of 4-aminobiphenyl-DNA adducts in M13 Replicative form (RF) DNA. M13 RF DNA was modified with N-OAc-TFAABP and replicated in both SOS-induced and uninduced cells. Transfectants were plated on indicator plates with M13 host cells. The number of transfectants, or infective centers, was enumerated to determine tranfection efficiency of ABP-modified genomes compared to unmodified genomes (buffer control conditions).


100
10
1
.1
Relative Survival (% of control)
0
20
40
60
80
100
Adducts /RF genome
DL7/p +UV
DL7/p
DL7 +UV
DL7
DL6 +UV
DL6
DL7 -UV
DL7 +UV
DL7/pGW16 -UV
DL7/pGW16 +UV
DL6 -UV
DL6 +UV

than in *uvr*$^+$ cells. The results suggest that the *uvr*$^+$ excision repair system is active in the repair of ABP lesions in M13 RF DNA. The 37% survival was attained at approximately 90, 45 and 25 ABP adducts per RF genome in *uvr*$^+$/*mucAB*, *uvr*$^+$ and *uvrA* cells induced for SOS-mutagenic processing.

Survival of M13 ABP-Modified SS DNA

In comparison, ABP lesions in M13 ss DNA were more genotoxic than ABP adducts in ds DNA. Survival of ABP-adducted ss DNA was not enhanced in excision repair-proficient cells compared to excision repair-deficient cells; the low transfection efficiency of ABP-adducted genomes was not enhanced by SOS-induction of host cells (Figure 17). In *uvr*$^+$ cells induced for SOS functions, two adducts/ss genome reduced genome survival to 37% of control DNA. The results indicate that survival of the M13 ss genome containing 5 adducts was reduced to approximately 5% of the b.c. treated DNA sample in all cells assayed.

Mutagenicity of ABP-DNA Lesions in M13 Genomes

Mutagenesis of M13 ABP-Modified RF DNA

The mutation frequency of M13 ABP-modified DNA was evaluated with genomes adducted with 0, 45 and 93 adducts/RF genome. There was a dose-response effect with increasing adduct level in both the DL7 (*uvr*$^+$) and the DL6 (*uvrA*) cells induced for SOS functions by UV-irradiation (Figure 18). The mutation frequency of ds DNA increased with dose level from 0 to 93

Figure 17.

Genotoxicity of 4-aminobiphenyl-DNA adducts in M13mp10 single-stranded (ss) DNA.. M13 ss DNA was modified with N-OAc-TFAABP and replicated in both SOS-induced and uninduced cells. Transfectants were plated on indicator plates with M13 host cells. The number of transfectants, or infective centers, was enumerated to determine tranfection efficiency of ABP-modified genomes compared to unmodified genomes (buffer control conditions).

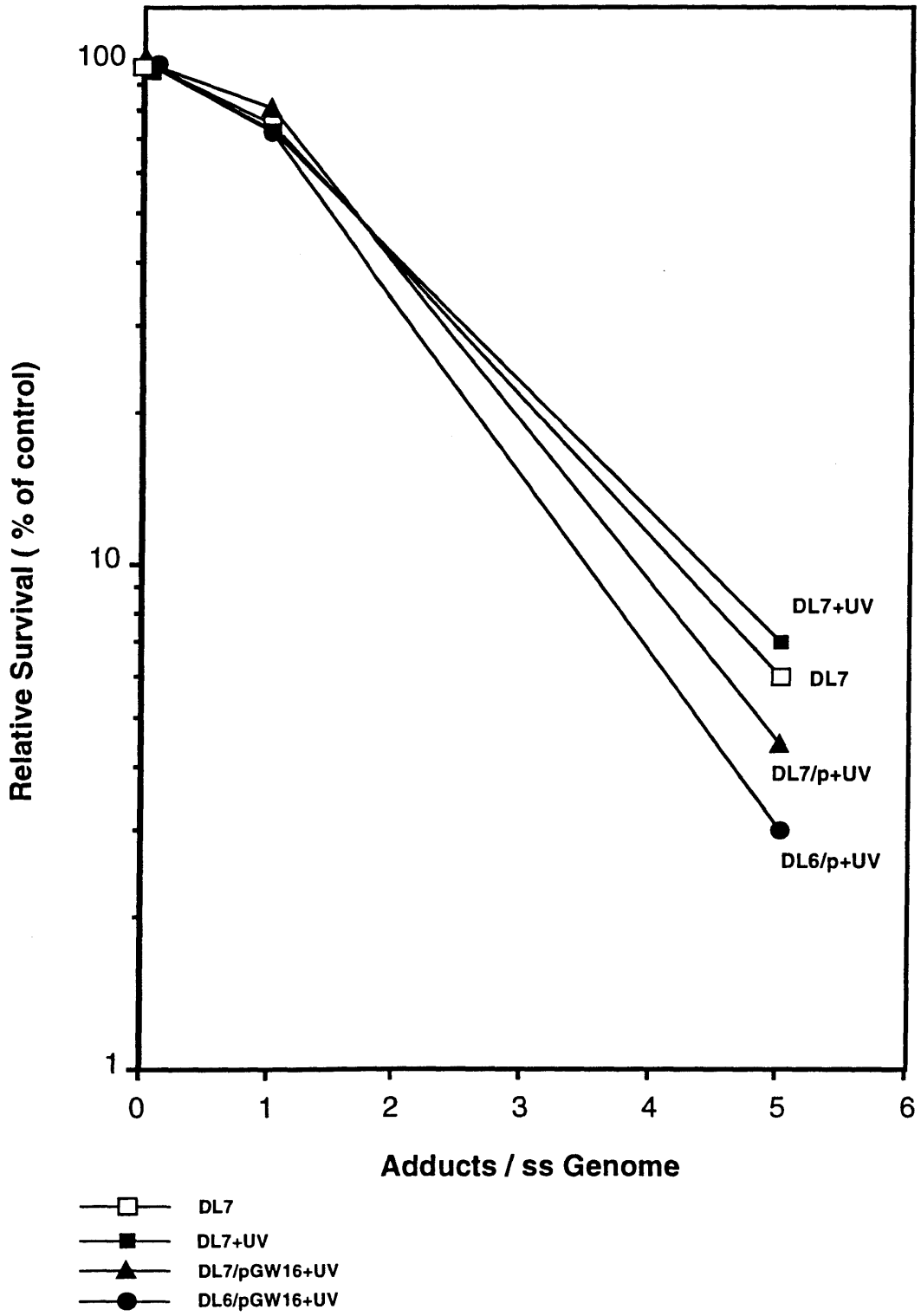
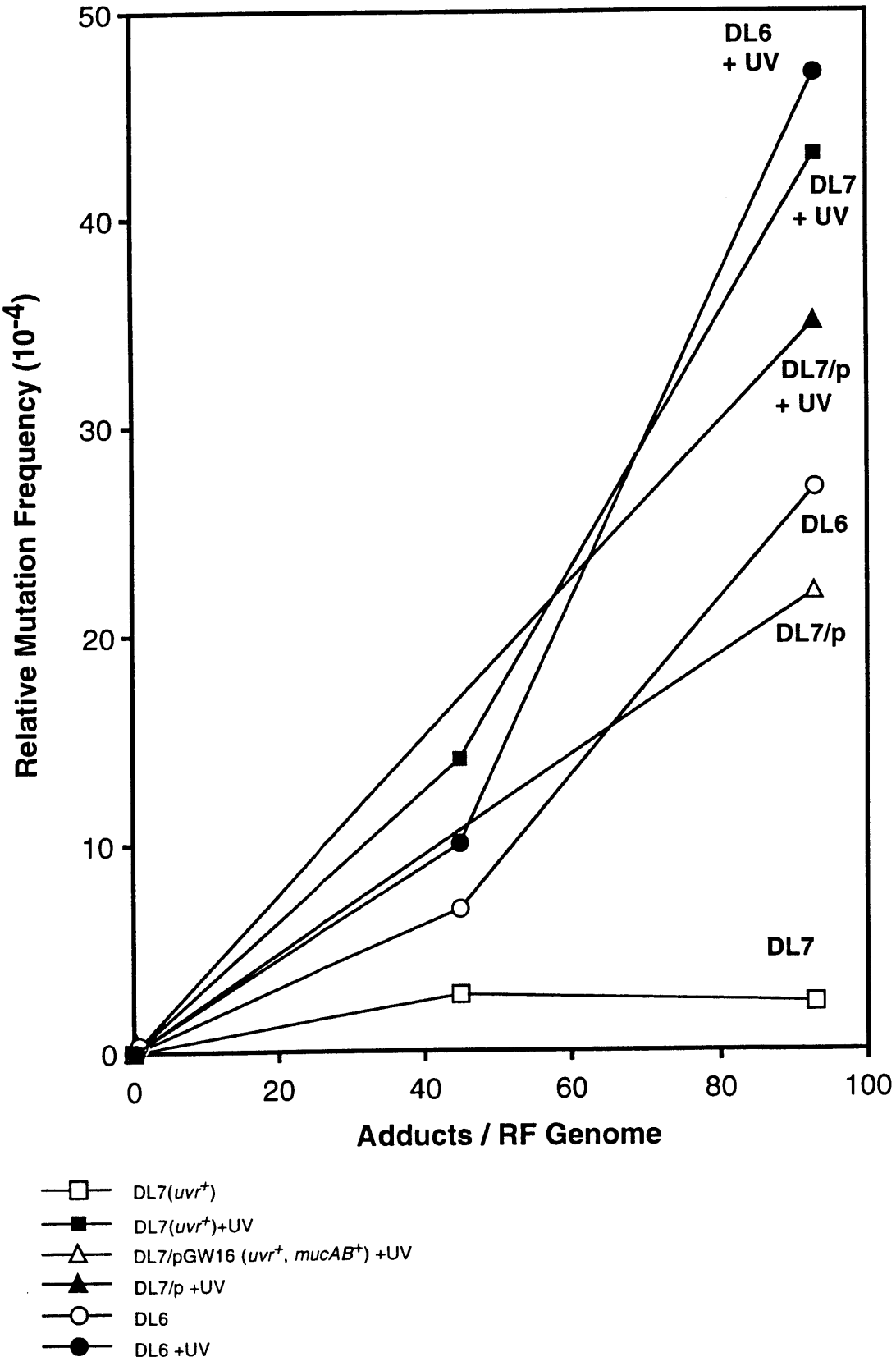

100
10
1
0
1
2
3
4
5
6
Relative Survival (% of control)
Adducts / ss Genome
DL7+UV
DL7
DL7/p+UV
DL6/p+UV
DL7
DL7+UV
DL7/pGW16+UV
DL6/pGW16+UV

Figure 18.

Mutagenesis induced by 4-aminobiphenyl DNA adducts in M13 Replicative form (RF) DNA. M13 RF DNA was modified with N-OAc-TFAABP and replicated in both SOS-induced and uninduced cells. Transfectants were plated on indicator plates with M13 host cells. The number of transfectants, or infective centers, was enumerated to determine mutation frequency of ABP-modified genomes compared to unmodified genomes. The mutation frequency (MF) is calculated by dividing the mutant plaques over the total number of plaques. The relative mutation frequency is expressed as the mutagenesis detected above background (buffer control conditions).


50
40
30
20
10
0
Relative Mutation Frequency (10^{-4})
0
20
40
60
80
100
Adducts / RF Genome
DL6
+ UV
DL7
+ UV
DL7/p
+ UV
DL6
DL7/p
DL7
DL7(uvr^+)
DL7(uvr^+)+UV
DL7/pGW16 (uvr^+, $mucAB^+$) +UV
DL7/p +UV
DL6
DL6 +UV

adducts/genome to 40-fold over background; (b.c. conditions, MF = 1.1 x 10^{-4}) in UV-irradiated DL7 cells (Table 3). In DL7 cells without SOS-induction there was no significant increase in mutagenesis above background. The results clearly indicate that in DL7 cells, mutagenesis induced by ABP lesions is dependent on the SOS mutagenic processing of the adducts.

In DL7/pGW16 (*uvr*$^{+}$/*mucAB*) cells, there was a 20-fold increase above background (b.c.) for RF genomes with 93 ABP adducts per genome; this value increased to 35-fold above background with SOS-induction of host cells. In DL6 cells, the mutation frequency of ABP-modified RF DNA increased with increasing number of ABP adducts, a significant increase in mutation frequency was observed in uninduced cells and this value increased with SOS induction of the host cell.

The mutation frequency of the "average" ABP adduct in the *lacZ* gene of RF DNA was determined to be approximately 0.09% in SOS-induced DL7/pGW16 cells. This value was calculated as follows (from Lasko et al., 1988): From Table 3, the relative mutation frequency induced by an RF genome with 93 adducts replicated in DL7/pGW16 cells (+UV), is 35 x 10^{-4}. The M13mp10 RF genome is approximately 7244 bp; therefore, 93 adducts would be distributed around the genome approximately every 78 bp. The M13 *lacZ* gene target is approximately 300 bp (Figure 11); therefore, the mean number of potential ABP adducts in the *lacZ* gene is approximately 4 adducts per target. The mutation frequency of the genome (93/RF) in SOS-induced cells, 35 x 10^{-4}, is divided by 4 adducts per target gene to yield, 8.8 x 10^{-4}, or a 0.09% mutation frequency for the average ABP lesion in the *lacZ* gene of RF DNA.

Table 3.

Mutagenesis by 4-aminobiphenyl in *E. coli*

in the lacZ gene of ABP-modified M13 RF (ds) DNA

Cells	+/- UV	DNA adducts/genome	MF[a] (10^{-4})	infective centers (mutants/total)	Relative MF[b] (10^{-4})	Survival % control
DL7 (*uvr*$^+$)	- UV	0	3.8	(3/7883)	0	100%
		45	6.5	(4/6114)	2.7	26%
		93	6.1	(2/3272)	2.3	7.0%
DL7 (*uvr*$^+$)	+ UV	0	3.8	(2/5182)	0	100%
		45	18	(10/5617)	14	35%
		93	47	(25/5325)	43	17%
DL7 /pGW16 (*uvr*$^+$)	- UV	0	1.0	(2/20,080)	0	100%
		45		n.d.	n.d.	n.d.
		93	23	(25/10,750)	22	31%
DL7 /pGW16 (*uvr*$^+$)	+ UV	0	1.3	(2/14,900)	0	100%
		45	n.d	n.d.	n.d.	n.d.
		93	36	(60/16630)	35	46%
DL6 (*uvrA*)	- UV	0	0.7	(1/15001)	0	100%
		45	7.6	(4/5294)	6.9	11%
		93	28	(1/352)	27	0.2%
DL6 (*uvrA*)	+ UV	0	2.6	(4/15504)	0	100%
		45	13	(10/7680)	10	17%
		93	50	(3/598)	47	0.3%

[a] Mutation frequency was determined by dividing the number of mutant plaques by the total number of plaques (infective centers)

[b] Relative mutation frequency is the MF above background

n.d.. not determined

Mutagenesis of M13 ABP-Modified SS DNA

ABP adducts in M13 ss DNA were observed to more mutagenic than in ds DNA and the frequency of mutations increased with SOS induction of the host cell (Figure 19). In DL7 cells that were not UV-irradiated there was only a two-fold increase in mutation frequency above background. The mutation frequency of modified ss DNA with 5 adducts per genome increased to about 4-fold above background (3.8×10^{-3}) in SOS-induced DL7 cells (Table 4). The greatest increase in mutation frequency was observed in UV treated DL7 cells containing the plasmid, pGW16. In SOS-induced DL7/pGW16 cells, the mutation frequency increased to approximately 12-fold above background (b.c. conditions). In DL6/pGW16 cells induced for SOS functions, the increase in mutagenesis was 14-fold above background. Overall, the results indicate a strong requirement for SOS mutagenesis gene functions in ABP-induced mutagenesis.

The mutagenic efficiency of the "average" ABP adduct in the *lacZ* gene was calculated to be approximately 6.2% in SOS-induced DL7/pGW16 cells. This value was calculated as follows (Lasko et al., 1988). From Table 4, the relative mutation frequency for an M13 ss genome with 5 adducts/ss genome was 12.4×10^{-3} in SOS-induced DL7/pGW16 cells. An ss genome with 5 adducts would have a mean number of 0.2 adducts per 300 b of the *lacZ* gene target. By dividing 12.4×10^{-3} by 0.2, I arrived at a mutation frequency of 6.2×10^{-2}, or approximately 6.2% for the average ABP lesion in ss DNA.

Figure 19.

Mutagenesis induced by 4-aminobiphenyl DNA adducts in M13 single-stranded (ss) DNA. M13 ss DNA was modified with N-OAc-TFAABP and replicated in both SOS-induced and uninduced cells. Transfectants were plated on indicator plates with M13 host cells. The number of transfectants, or infective centers, was enumerated to determine mutation frequency of ABP-modified genomes compared to unmodified genomes. The mutation frequency (MF) is calculated by dividing the mutant plaques over the total number of plaques. The relative mutation frequency is expressed as the mutagenesis detected above background (buffer control conditions).

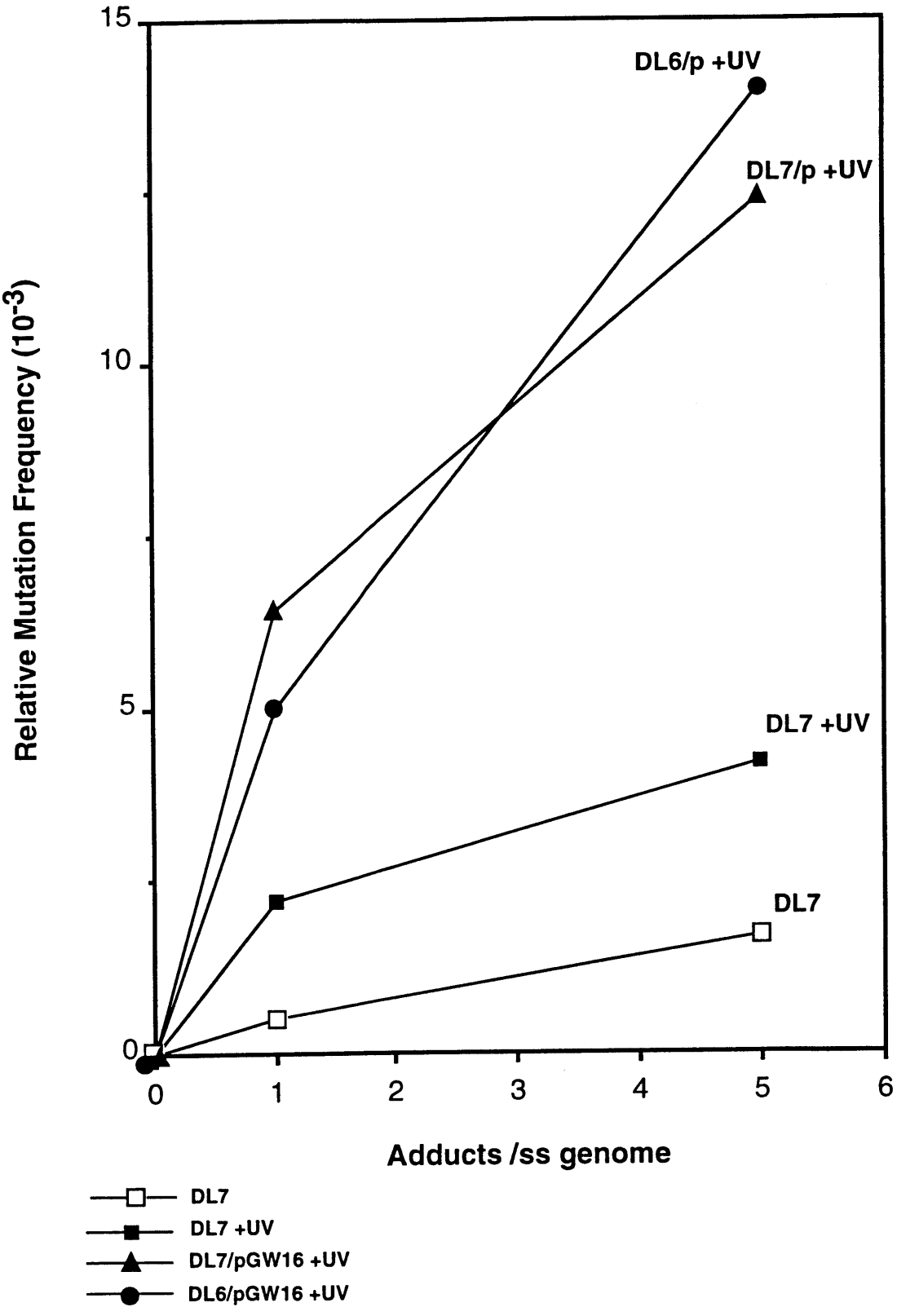

15
10
5
0
Relative Mutation Frequency (10^{-3})
DL6/p +UV
DL7/p +UV
DL7 +UV
DL7
0
1
2
3
4
5
6
Adducts /ss genome
DL7
DL7 +UV
DL7/pGW16 +UV
DL6/pGW16 +UV

Table 4.

Mutagenesis by 4-aminobiphenyl in *E. coli*

in the lacZ gene of ABP-modified M13 ss DNA

Cells	+/- UV	DNA adducts/ genome	MF [a] (10^{-3})	infective centers mutants/ total	Relative MF [b] (10^{-3})	Survival % control
		0	2.5	(40/13990)	0	100%
DL7 (*uvr*$^+$)	- UV	1	3.4	(37/9269)	0.5	75%
		5	4.2	(66/16489)	1.7	6.0%
		0	3.8	(52/14263)	0	100%
DL7 (*uvr*$^+$)	+ UV	1	6.0	(38/2988)	2.2	73%
		5	8.0	(85/10590)	4.2	7.0%
		0	8.6	(51/5950)	0	100%
DL7 /pGW16	+ UV	1	15	(83/6084)	6.4	81%
(*uvr*$^+$/ *mucAB*)		5	21	(93/4344)	12.4	4.4%
		0	7.0	(14/2022)	0	100%
DL6 /pGW16	+ UV	1	12	(21/1739)	5.0	72%
(*uvrA*)		5	21	(20/958)	14	3.0%

[a] Mutation frequency (MF) is expressed as the number of mutant plaques divided by the total number of plaques (infective centers)

[b] Relative mutation frequency (MF) is expressed as the MF above background

Spectrum of Mutations Induced by ABP-DNA Adducts in the *lacZα* Gene of M13 SS DNA

Once the mutation frequencies were determined, independent mutants were isolated and restreaked to confirm plaque phenotype. M13 ss DNA was prepared from single plaques and sequenced to evaluate the types and positions of the mutations in the *lacZ* gene. The sequencing data indicate that in ss DNA, ABP induces a spectrum of mutational changes predominantly base-substitution mutations at deoxyguanosine bases (Table 5). The mutations induced in SOS-induced DL7 (uvr^+) or DL7/pGW16 (uvr^+/*mucAB*) cells were similar to those found in uninduced DL7 cells, albeit at a higher occurrence.

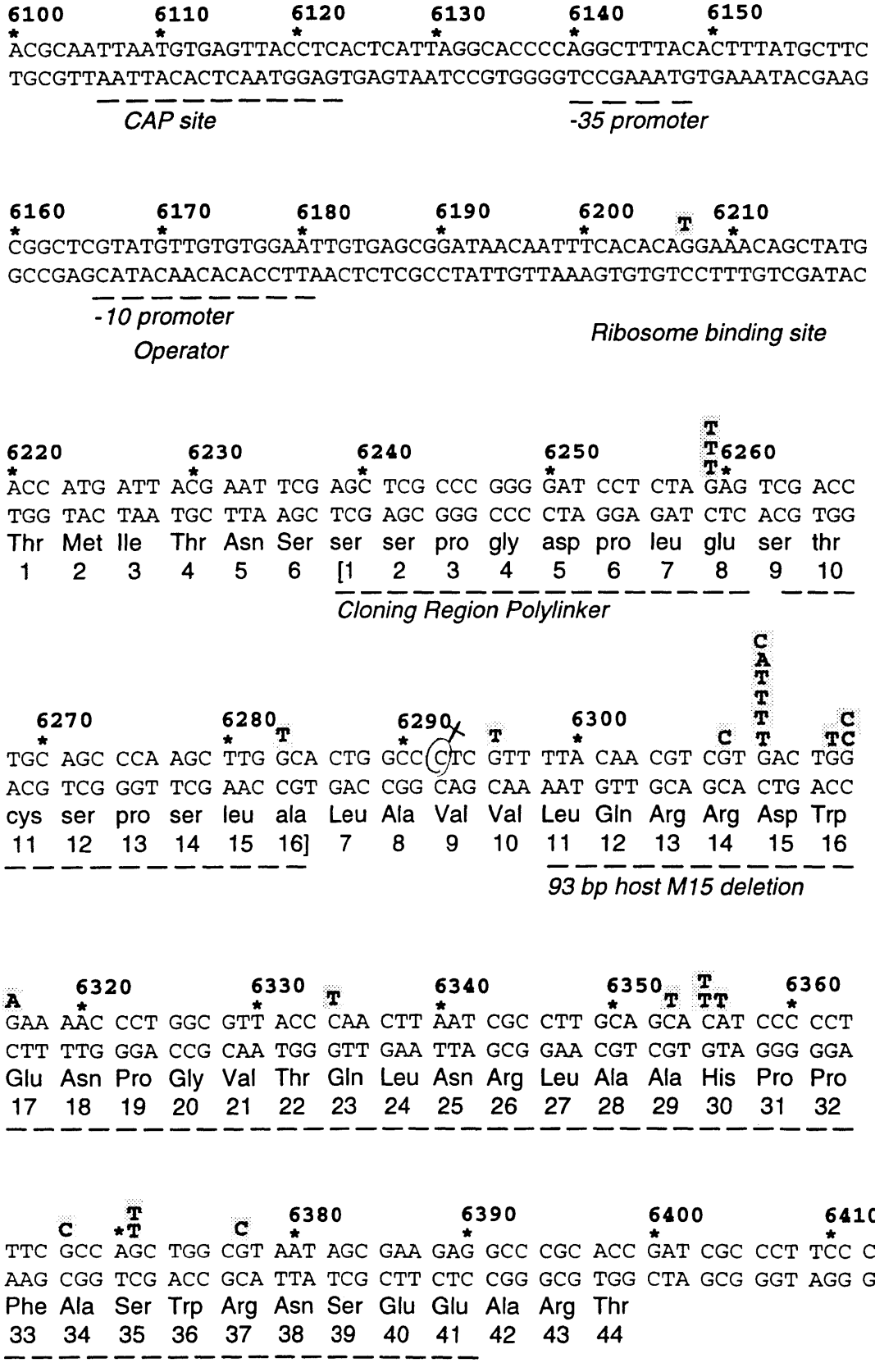
The partial mutational spectrum formulated for ABP in the *lac*Z gene of M13 ss DNA revealed that ABP-induced mutations in ss DNA were similar to those previously found in ds DNA (Lasko et al., 1988) with two common hotspots of mutation for ss and ds DNA in the *lac*Z gene; over 50% of the base-substitution mutations were found in these sequences (Figure 20). The dG at the position 6310 in the sequence, 5'-TGA-3', was found to be the site of mutation for approximately 25% of the observed base changes in ss DNA. Among the base changes in ss DNA, 80% occurred at putative dG adducts (we assume that mutagenesis is targeted to the site of the adduct, but we have no substantiating evidence). In SOS-induced DL7 and DL7/pGW16 cells, the primary mutation observed was the G -> T transversion; however, in uninduced DL7 cells, both G -> T and G -> C mutations occurred with equal frequency (Table 5).

Table 5.
Partial mutational spectrum for 4-aminobiphenyl
in the lacZ gene of M13 ss ABP-DNA

	Mutation	Occurrences	Location	Sequence
- SOS	G -> T	1	6295	TC**G**TT
	G -> T	1	6310	GT**G**AC
	G -> T	1	6371	CA**G**CT
	G -> A	1	6310	GT**G**AC
	G -> C	2	6315	TG**G**GA
	G -> C	1	6377	GC**G**TA
	C -> T	1	6353	AG**C**AC
	C -> T	1	6355	CA**C**AT
+SOS	G -> T	1	6207	CA**G**GA
	G -> T	3	6259	TA**G**AG
	G -> T	1	6283	TG**G**CA
	G -> T	3	6310	GT**G**AC
	G -> T	1	6314	CT**G**GG
	G -> T	1	6371	CA**G**CT
	G -> C	1	6308	TC**G**TG
	G -> C	1	6310	GT**G**AC
	G -> C	1	6367	TC**G**CC
	G -> A	1	6316	GG**G**AA
	A -> T	1	6356	AC**A**TC
	C -> T	1	6334	CC**C**AA
	C -> T	1	6355	CA**C**AT

Figure 20.

Mutations induced by 4-aminobiphenyl in the *lacZ* gene of M13mp10 single-stranded DNA. Base substitution mutations observed in the *lacZ* gene are shown in the shaded boxes above the gene sequence.


6100 6110 6120 6130 6140 6150
ACGCAATTAATGTGAGTTACCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTC
TGCGTTAATTACACTCAATGGAGTGAGTAATCCGTGGGGTCCGAAATGTGAAATACGAAG
CAP site
-35 promoter
6160 6170 6180 6190 6200 6210
CGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATG
GCCGAGCATACAACACACCTTAACTCTCGCCTATTGTTAAAGTGTGTCCTTTGTCGATAC
-10 promoter
Operator
Ribosome binding site
6220 6230 6240 6250 6260
ACC ATG ATT ACG AAT TCG AGC TCG CCC GGG GAT CCT CTA GAG TCG ACC
TGG TAC TAA TGC TTA AGC TCG AGC GGG CCC CTA GGA GAT CTC ACG TGG
Thr Met Ile Thr Asn Ser ser ser pro gly asp pro leu glu ser thr
1 2 3 4 5 6 [1 2 3 4 5 6 7 8 9 10
Cloning Region Polylinker
6270 6280 6290 6300
TGC AGC CCA AGC TTG GCA CTG GCC CTC GTT TTA CAA CGT CGT GAC TGG
ACG TCG GGT TCG AAC CGT GAC CGG CAG CAA AAT GTT GCA GCA CTG ACC
cys ser pro ser leu ala Leu Ala Val Val Leu Gln Arg Arg Asp Trp
11 12 13 14 15 16] 7 8 9 10 11 12 13 14 15 16
93 bp host M15 deletion
6320 6330 6340 6350 6360
GAA AAC CCT GGC GTT ACC CAA CTT AAT CGC CTT GCA GCA CAT CCC CCT
CTT TTG GGA CCG CAA TGG GTT GAA TTA GCG GAA CGT CGT GTA GGG GGA
Glu Asn Pro Gly Val Thr Gln Leu Asn Arg Leu Ala Ala His Pro Pro
17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
6380 6390 6400 6410
TTC GCC AGC TGG CGT AAT AGC GAA GAG GCC CGC ACC GAT CGC CCT TCC CAA CAG
AAG CGG TCG ACC GCA TTA TCG CTT CTC CGG GCG TGG CTA GCG GGT AGG GTT GTC
Phe Ala Ser Trp Arg Asn Ser Glu Glu Ala Arg Thr
33 34 35 36 37 38 39 40 41 42 43 44

Site-Specific Mutagenesis Studies with Genomes Containing the Major ABP-DNA Adduct

The initial site-specific mutagenesis studies that I conducted with M13 genomes were made with an ABP-adducted tetranucleotide, 5'-TG-(ABP)CA-3', synthesized at NCTR. The synthesis and characterization of the tetranucleotide used for my initial experiments was described by Lasko et al. (1987). Throughout this section, I shall refer to the ABP-tetranucleotide used in these initial studies as TG-(ABP)CA (NCTR).

A site-specifically modified genome was constructed by ligating either the modified tetranucleotide, 5'-TG-(ABP)CA-3'(NCTR), or the corresponding unmodified tetramer, 5'-TGCA-3' into a unique *Pst*I site restriction enzyme site in a M13 gapped heteroduplex DNA vector. After its construction, the ABP-modified genome was characterized to map the location of the ABP-DNA adduct in the vector. The results of the *Pst*I restriction enzyme digestion assay, used as a criterion to assess the purity of the ABP-modified genome, indicated that the ABP-tetranucleotide may have undergone some degradation since its synthesis at NCTR.

Subsequently, the ABP-genomes were digested with *Pst*I enzyme prior to transfection into *E. coli*, assuming the treatment would eliminate possible contaminants present in the sample. Transfection assays with site-specifically ABP-modified and unmodified genomes were carried out in DL7 cells and SOS-induced DL7 and DL7/pGW16 cells. Mutants were isolated and the mutation frequency as well as the types of mutations induced by the ABP lesion in M13 ss DNA were determined. Although, the mutagenesis results and sequencing data obtained with the site-specifically modified genome

were consistent with the data from the mutational spectrum observed in the *lacZ* gene of globally ABP-modified ss genomes, it was important to confirm the these experiments with a pure, freshly-prepared ABP-modified tetranucleotide. Hence, a new ABP-adducted tetranucleotide, designated as TG-(ABP)CA (MIT), was synthesized and characterized in our laboratory.

In the first section of the RESULTS chapter I will present the results from studies conducted with the newly-synthesized, TG-(ABP)CA (MIT). Subsequently, the analysis of site-specifically ABP-modified genomes constructed with TG-(ABP)CA (MIT), the transfection of the modified and the control genomes into *E. coli* cells, and the mutant selection protocols are described in the following sections. Finally, in the last part of the RESULTS chapter, I will describe the results of my initial mutagenesis experiments conducted with M13 genomes made with the NCTR ABP-adducted tetranucleotide.

I have presented the results from my experiments in this order because I have the most confidence in the newly-synthesized and well-characterized tetranucleotide, TG-(ABP)CA (MIT), and I am less confident in the purity of the TG-(ABP)CA (NCTR) (based on HPLC analysis of TG-(ABPCA (NCTR) and characterization of genomes containing this tetranucleotide). The coincidence, however, between the type of mutations induced by the NCTR ABP tetranucleotide and the predominant mutational event observed in the known mutational spectrum of ABP, as established in our laboratory, may be significant; therefore, I have included the results from my initial mutagenesis assays in this dissertation.

Synthesis, Purification and Characterization of the Deoxytetranucleotide, 5'-d[TpG-(ABP)pCpA]-3'(MIT), Containing the Major DNA Adduct of ABP

HPLC Analysis of the Synthesis and Purification of d[pTpG-(ABP)pCpA] (MIT)

The tetranucleotide 5'-d[TpGpCpA]-3' was synthesized and allowed to react with *N*-acetoxy-4-aminobiphenyl (*N*-OAc-ABP), an electrophilic derivative of ABP to produce 5'-d[TpG-(ABP)pCpA]-3' (MIT). Analysis of the synthesis by reversed-phased HPLC indicated that the yield of d[TG-(ABP)CA] (MIT) was approximately 30-40% from the reaction of TGCA with *N*-OAc-ABP (Figure 21 a,b).

The unadducted and ABP-adducted oligonucleotides were purified by a reversed-phase HPLC system and characterized by UV spectroscopy. The UV spectrum of TG-(ABP)CA (MIT) was shown to be different from that of TGCA; an absorbance shoulder was present at 310 nm in the spectrum, typical of C8-arylamine-substituted nucleotides (Figure 22).

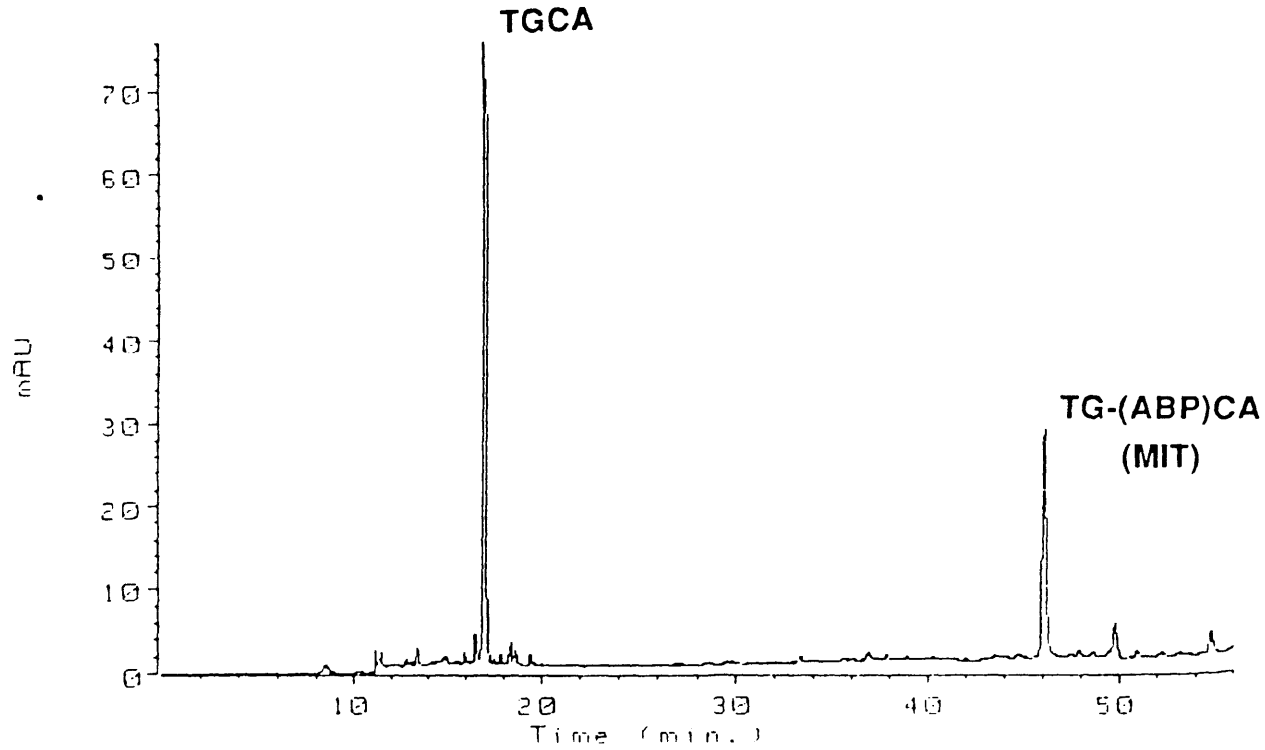
Characterization of d[pTpG-(ABP)pCpA] (MIT) by Mass Spectrometry

In collaboration with Dr. C. Costello (Chemistry department, MIT), fast-atom bombardment (FAB) mass spectrometry was used to analyze the structural characteristics of the TG-(ABP)CA (MIT) tetranucleotide. The TG-(ABP)CA oligonucleotide was calculated to have a molecular weight of 1340; indeed the negative ion spectrum for TG-(ABP)CA showed the major $[M - H]^-$ ion to be at *m/z* 1339, the expected value.

Figure 21.

a. HPLC analysis of the crude reaction mixture from the adduction of 5'-TGCA'3' with N-acetoxy-4-aminobiphenyl (N-OAc-ABP), to produce 5'-TG-(ABP)CA-3'. b. HPLC analysis of the purified product, 5'-TG-(ABP)CA-3'.

a) Crude Synthesis Mixture: TGCA and TG-(ABP)CA



b) Purified Product: TG-(ABP)CA

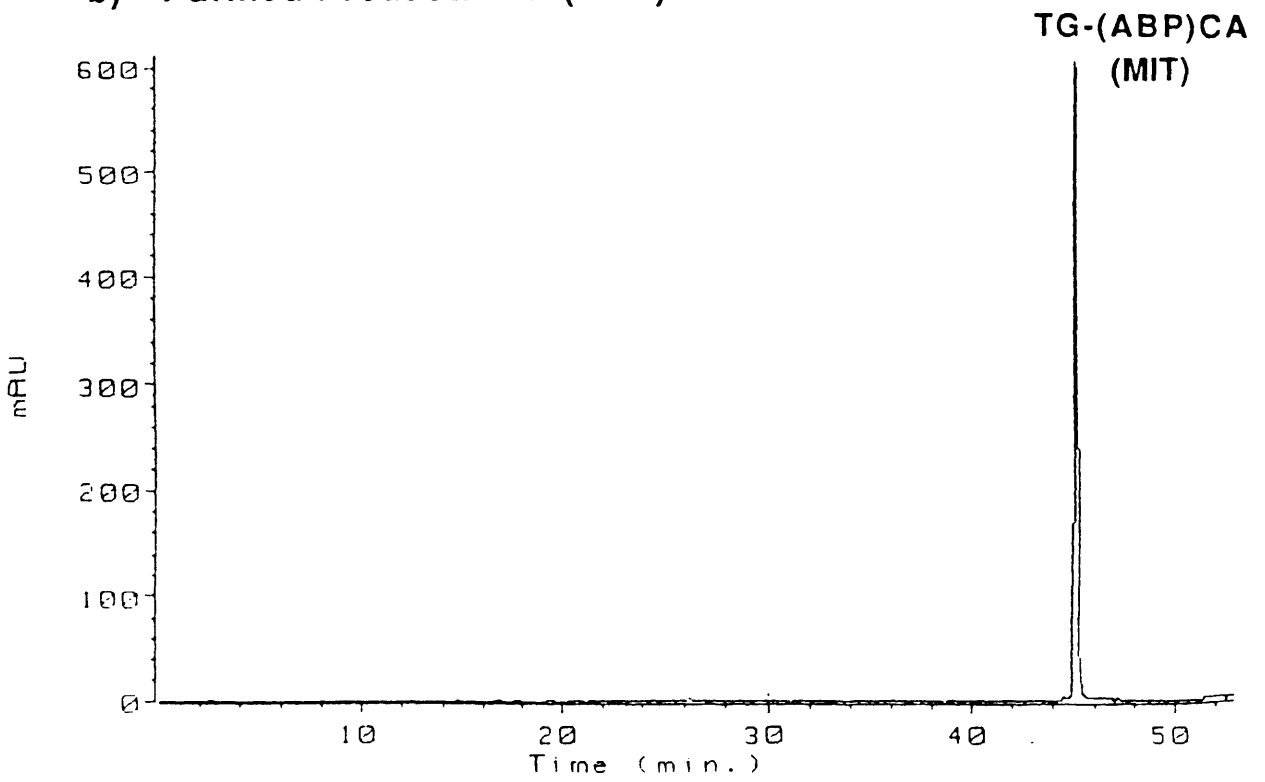
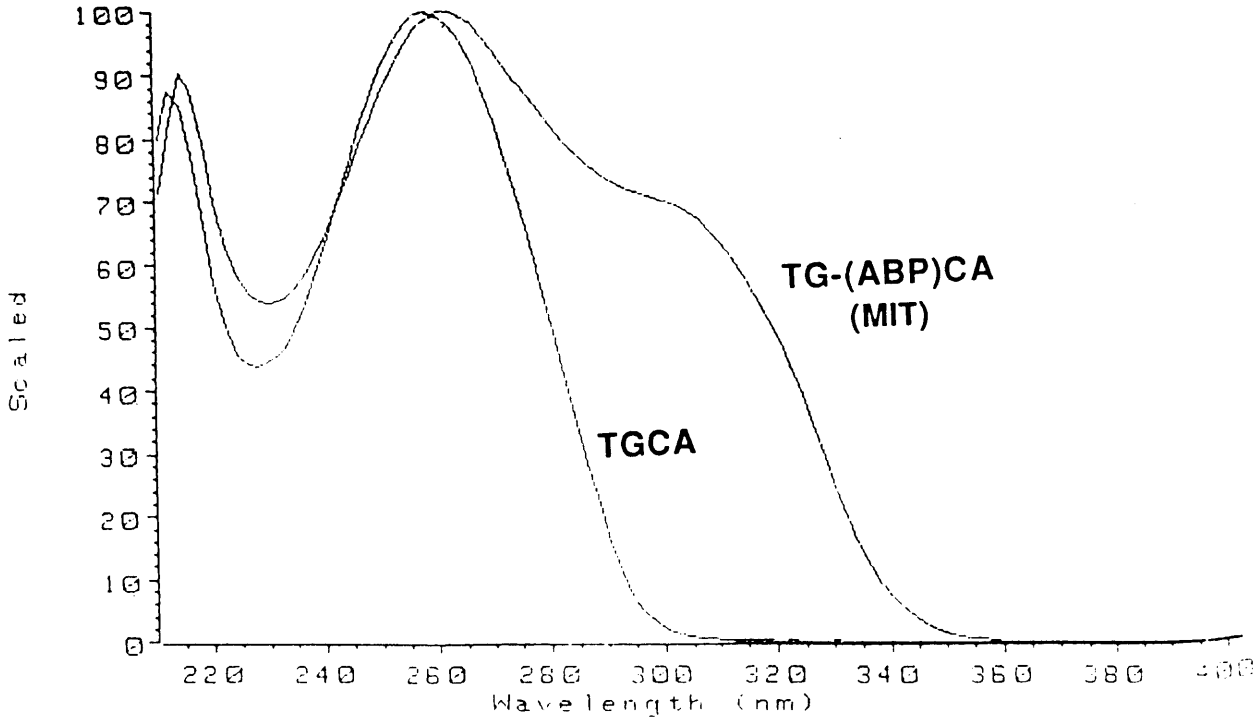


Figure 22.

UV spectra of the tetranucleotides, TGCA and TG-(ABP)CA. An absorbance shoulder at 310 nm in the spectrum for TG-(ABP)CA is observed, typical of C8-arylamine-substituted nucleotides (Lasko et al., 1987).

UV spectra of TGCA and TG-(ABP)CA



Nucleoside Composition Analysis of d[pTpG-(ABP)pCpA] (MIT)

The ABP-modified and unmodified tetramers were enzymatically digested to their respective nucleosides, which were then analyzed by HPLC to confirm the nucleoside composition. Digestions by nuclease P_1 and snake venom phosphodiesterase were conducted on the unmodified tetranucleotide, d[TpGpCpA] and the ABP-modified tetramer d[TpG-(ABP)pCpA] (MIT) in parallel. An analysis of the digestion products on reversed-phase HPLC indicated that the enzymatic digestion had gone to completion (Figure 23a). The four peaks that were observed for TGCA occurred in equimolar ratio and corresponded to the nucleosides dC, dG, deoxythymidine (dT) and dA. The chromatogram of the digested tetramer, dTG-(ABP)CA (MIT), exhibited no dG peak but instead a prominent peak with a retention time identical to that of an authentic dG-(ABP) standard (Figure 23b).

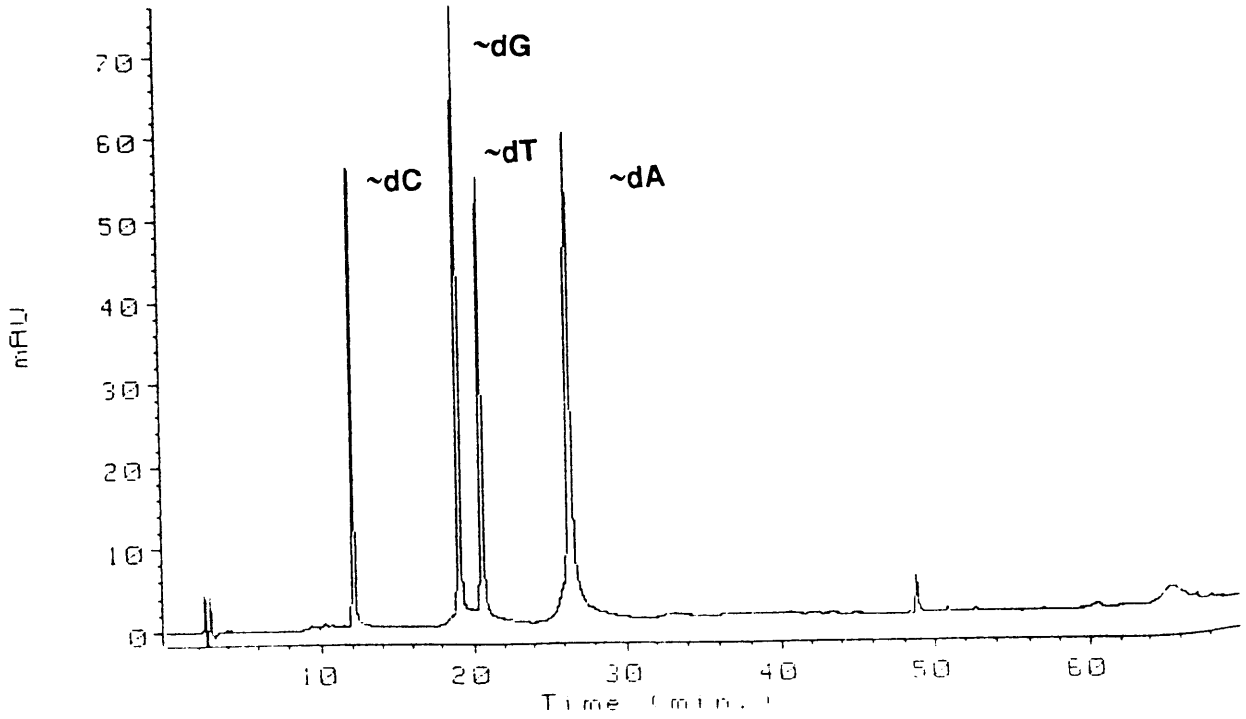
Electrophoretic Gel Analysis of d[pTpG-(ABP)pCpA] (MIT)

In separate reactions the ABP-modified and unmodified oligonucleotides were subjected to the same heat denaturation conditions (100°C, 5 min) used to produce the ss genomes used for the mutagenesis assays. Both tetranucleotides appeared not to have undergone any chemical degradation when analyzed by HPLC and UV spectroscopy. In addition, both the unmodified and ABP-modified tetranucleotides were shown to be pure when 5'-end-labeled with [γ^{32}P]-ATP and analyzed on a 23% acrylamide denaturing gel. After purification and characterization of TG-(ABP)CA (MIT) and TGCA, the phosphorylated tetranucleotides were ligated into M13 gapped duplex

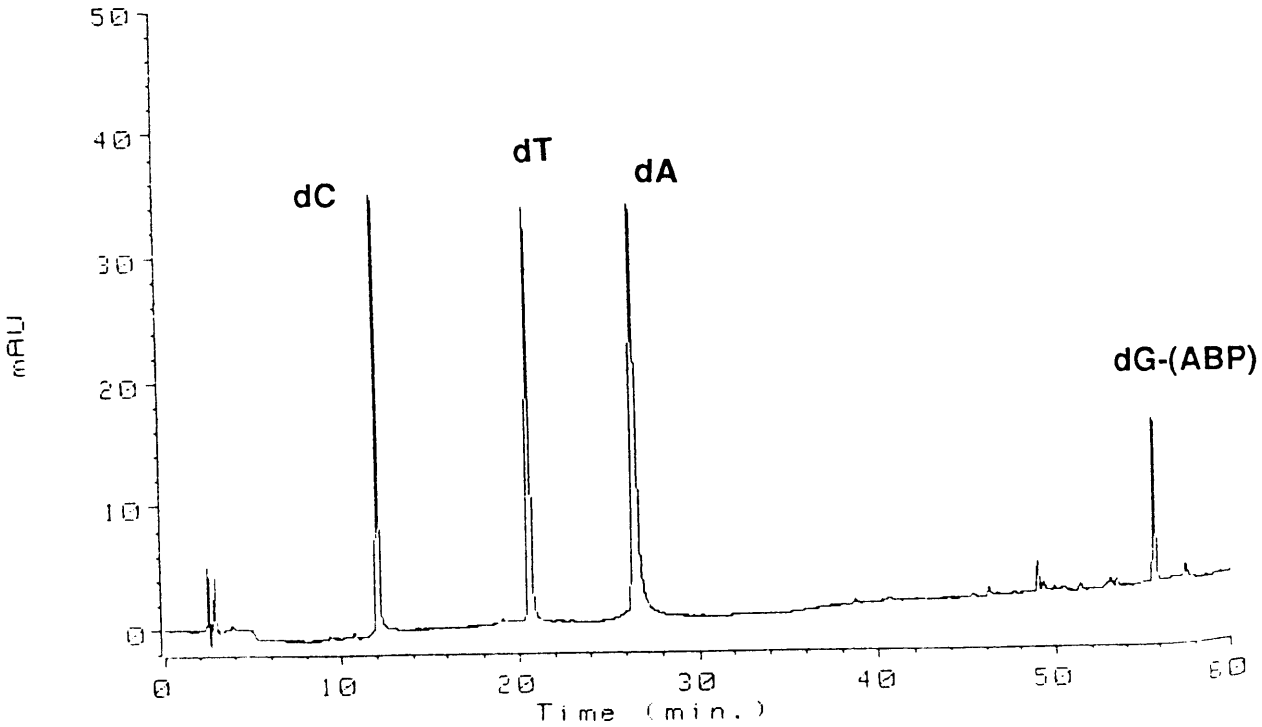
Figure 23.

Nucleoside composition analysis of the tetranucleotides. a. HPLC analysis of the digestion of TGCA with nuclease P_1 and snake venom phosphodiesterase produced four peaks, dT, dG, dC, and dA. b. HPLC analysis of the parallel enzymatic digestion of TG-(ABP)CA, yielded four peaks, dT, dC, dA and dG-C8-ABP.

a) TGCA digestion



b) TG-(ABP)CA (MIT) digestion



vectors to produce the site-specifically ABP-modified and unmodified genomes, respectively.

Characterization of an M13 Genome Containing a Single ABP-DNA Lesion

A site-specifically modified M13 genome containing the dG-C8-ABP adduct at a unique *Pst* I site, 5'-CTGCAG-3', was constructed and characterized (the adduct was situated at the underscored base). According to the protocol, genomes were produced containing the adduct in this palindromic sequence in either the plus strand of the M13 genome at position 6245, or in the minus strand at position 6246. The site-specifically modified genome is designated as M13-TG-(ABP)CA (MIT) and the control, unmodified genome as M13-TGCA (Figure 13).

Estimation of Ligation Efficiency of the ABP-Modified Genome

The efficiency of ligation of the adducted tetramer into the gapped duplex genomes was calculated by determining the specific activity (in cpm/pmol) of the tetramer and comparing it to the specific activity of the ligated genomes after purification. Several calculations for ligation were performed, after which it was determined that the ligation efficiency of the adducted tetranucleotide into the gapped heteroduplex vector was approximately 25% compared to a 50% ligation efficiency for the unmodified genome, M13-TGCA. The lower ligation efficiency observed with M13-TG-(ABP)CA (MIT) could be due to the presence of the bulky ABP lesion in a tetranucleotide sequence. In comparison, ligation of a tetranucleotide containing an a minor O^6-methlyguanine adduct occurred with an efficiency of 50% into an M13 vector at the *Pst*I site (Green et al., 1984).

Restriction Enzyme Analysis of the ABP-Modified Genomes

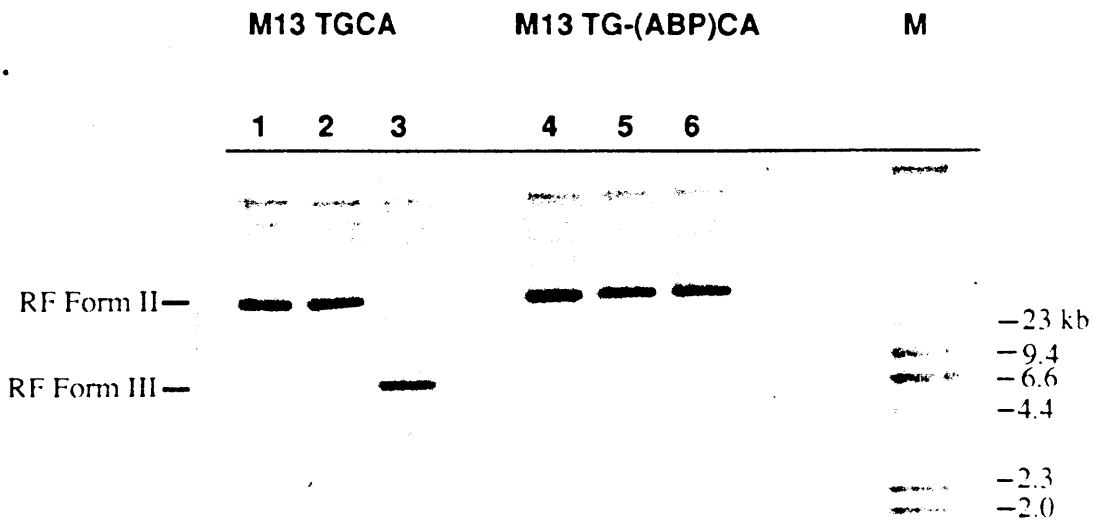
Both of the ligated genomes, M13TG-(ABP)CA (MIT) and M13-TGCA, were characterized by *Pst*I restriction enzyme digestion to confirm the location of the adduct in the genome as well as to observe if the presence of the adduct rendered the genome refractory to cleavage by the *Pst*I enzyme. The adduct resided within the *Pst*I site and hence failure of the modified genome to cleave was used as a criterion to assess the purity of the singly-adducted genome. The *Pst*I enzyme restriction analysis of both the ABP-modified and control genomes is shown in Figure 24. The characterization experiments demonstrated that M13 genomes constructed with the ABP-tetramer were resistant to enzymatic digestion by *Pst*I. As expected, the unmodified genome was linearized with restriction by *Pst*I. These results correlate with earlier studies in which the presence of an ABP-DNA adduct at the *Pst*I site in an M13 genome rendered the restriction enzyme incapable of cleavage at that site (Lasko et al., 1987).

5′ and 3′ Ligation Analysis of the ABP-Modified Genome

In addition to the *Pst*I digestion analysis, it was important to determine the actual percentage of constructed genomes in which ligation had occurred on both the 5′ and 3′ ends of the tetranucleotide since the nicked duplex genomes were to be denatured to ss DNA prior to transfection in *E. coli*. Restriction enzymes that cleaved the genomes in the vicinity of the *Pst*I site were used to digest both the adducted and the unadducted genomes. The results of the experiment indicated that greater than 95% of the both the ABP-modified and the unmodified genomes were ligated at both

Figure 24.

Restriction enzyme and gel analysis of the control genome, M13 TGCA (Lanes 1-3), and the site-specifically ABP-modified genome, M13 TG-(ABP)CA (Lanes 4-6). Ligated genomes were digested with *Pst*I enzyme to assess the purity of the genomes as well as to map the location of the adduct at the *Pst*I site. Undigested samples, buffer control (no enzyme), and *Pst*I treated samples were electrophoresed on 0.8% agarose gels containing ethidium bromide. Due to the nick in the genome at the *Bgl*II site in one strand of the genome, the uncut M13TGCA and the M13TG-(ABP)CA genomes migrate as RF form II (nicked circular) DNA under these conditions (Lanes 1 and 4, respectively). In addition, the buffer control samples also migrate as uncut DNA (Lanes 2 and 5, respectively). The unmodified M13TGCA genome is linearized with *Pst*I digestion, and hence migrates as RF form III (RF linear) DNA under these electrophoresis conditions (Lane 3). The presence of the ABP adduct in the M13TG-(ABP)CA genome, however, inhibits cleavage by *Pst*I and thus the modified genomes still migrate as form II DNA after enzyme treatment (Lane 6).


M13 TGCA
M13 TG-(ABP)CA
M
1 2 3 4 5 6
RF Form II
RF Form III
23 kb
9.4
6.6
4.4
2.3
2.0


Pst I site
6245 (+) or
6246 (–)
M13 TGCA
M13 TG-(ABP)CA
(7.2 kb)

the 5′ and 3′ sides of the tetranucleotide (Figure 25). This restriction analysis also mapped the ABP lesion to an 18 base restriction fragment in the M13 genome.

One important method of genome analysis was performed to estimate the actual amount of viable ss circular DNA present in the ligated genomes. When both the ABP-modified and the unmodified nicked duplex genomes were denatured with heat at 100°C and analyzed in agarose gels, it was determined that approximately 50% of the DNA was present as ss circular genomes (Figure 26). Although the 5′- 3′ ligation analysis indicated that > 95% of the DNA was ligated at both ends of tetramer, the lower amount of closed, circular ss DNA may be attributed to random nicks in other regions of the duplex genome.

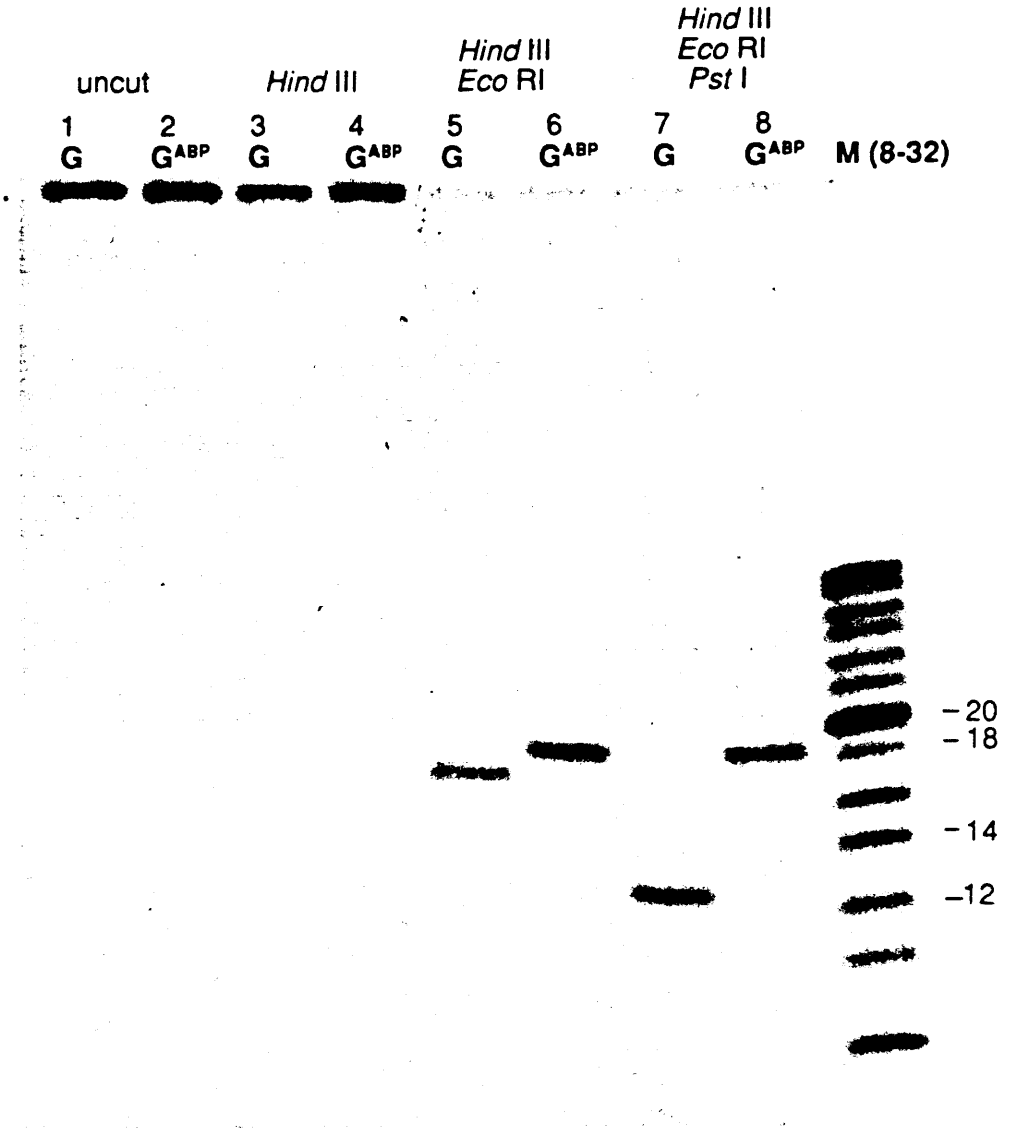
Genotoxicity and Mutagenicity of a Single ABP Lesion in an M13 SS Genome

Transfection of a Singly-Modified M13 Genome into E. coli

The site-specifically ABP-modified and control genomes used for the mutagenesis experiments were constructed prior to each experiment as described in the previous section. Both the genomes, M13-TG-(ABP)CA (MIT) and M13-TGCA, were transfected by the method of electroporation into *E. coli* DL7 (*uvr*$^+$) cells or SOS-induced *E. coli* DL7/pGW16 (*uvr*$^+$/*muc*AB) cells (Figure 14). The method of electrotransformation was used to transfect DNAs because it was important to obtain a large number of transfectants for accurate mutant analysis. The electroporation protocol could produce ~10^7 infective centers/μg of ss DNA compared to the standard

Figure 25.

5′ and 3′ ligation analysis of the site-specifically ABP-modified, M13-TG-(AB)CA (G^{ABP}), and the control, M13-TGCA (G) genomes. Ligated genomes were digested with the enzymes, *Hind*III, *Eco*RI, and *Pst*I, that cut within the local region of the adduct site to assess the extent of ligation on both the 5′ and the 3′ ends of the unmodified, TGCA, and the ABP-modified, TG-(ABP)CA, tetranucleotides into the M13GHD vectors. Digestion reactions were heat denatured and electrophoresed in 20% polyacrylamide, 7 M urea gels. The uncut and the singly digested G and G^{ABP} genomes are represented in Lanes 1-4. Efficient (100%) 5′-3′ ligation would produce an 18 b fragment for a double digestion with *Hind*III and *Eco*RI under these conditions (Lanes 5 and 6). 5′ ligation, but not 3′, would produce a 13 b fragment. 3′ ligation, but not 5′, would yield a 9 b fragment. Addition of *Pst*I to the *Hind*III/*Eco*Rl digestion reaction produces a 13 b fragment for M13TGCA, but, as expected, does not cleave the M13 TG-(ABP)CA genome (Lanes 7 and 8).


uncut
Hind III
Hind III
Eco RI
Hind III
Eco RI
Pst I
1 G
2 G^ABP
3 G
4 G^ABP
5 G
6 G^ABP
7 G
8 G^ABP
M (8-32)
–20
–18
–14
–12

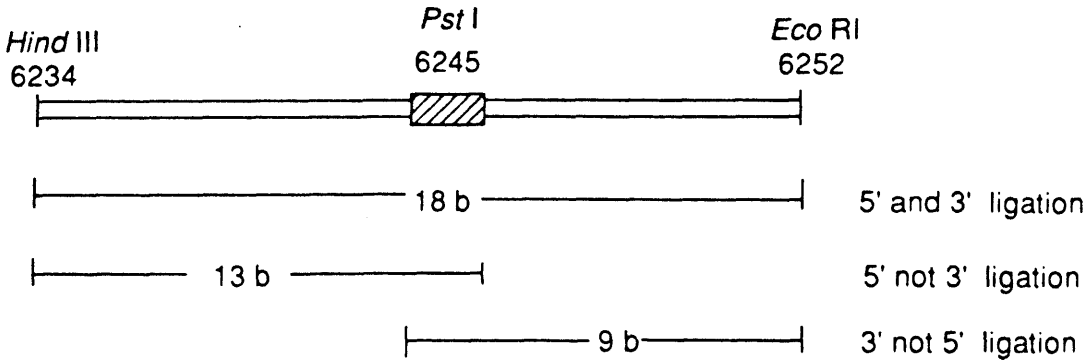
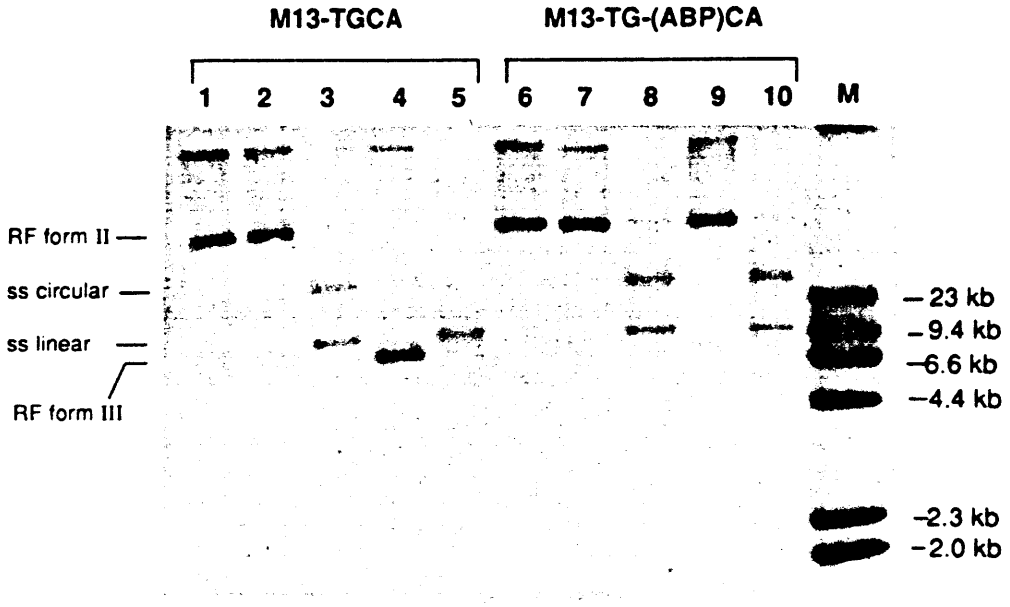

Hind III
6234
Pst I
6245
Eco RI
6252
18 b
5' and 3' ligation
13 b
5' not 3' ligation
9 b
3' not 5' ligation

Figure 26.

Estimation of the amount of viable site-specifically ABP-modified single-stranded genome and control single-stranded genome. ABP-modified, M13 TG-(ABP)CA, and control, M13 TGCA, genomes were heat denatured and analyzed on 1.0% agarose gels to determine the percent of ss circular DNA in each ligation sample. Uncut and buffer control M13TGCA and M13TG-(ABP)CA genomes migrate as RF form II (RF nicked circular) under these conditions (lanes 1, 6 and 2, 7, respectively). *Pst*I treatment of M13TGCA linearizes the genome to RF form III (RF linear) (lane 4); however, *Pst*I treatment of M13TG-(ABP)CA does not alter the migration of the genome (lane 9). After heat denaturation of the genomes two DNA forms, ss circular and ss linear, are visible for M13TGCA and M13TG-(ABP)CA (lanes 3 and 8, respectively). Heat denaturated *Pst*I digested M13TGCA and M13TG-(ABP)CA samples are shown in Lanes 5 and 10, respectively.


M13-TGCA
M13-TG-(ABP)CA
1
2
3
4
5
6
7
8
9
10
M
RF form II
ss circular
ss linear
RF form III
23 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb

$CaCl_2$ method of transformation, which produces only 10^4-10^5 transfectants/μg of ss DNA.

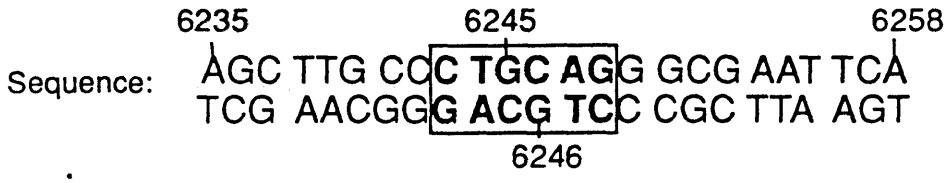
After transfection of the genomes, an aliquot of the mixture was plated on an M13 host strain, *E. coli* GW5100, to produce infective centers; these were enumerated to determine survival of modified genomes. Subsequently, progeny phage produced from the initial transfection (T_1) were plated and enumerated to determine the number of progeny phage produced after T_1.

Protocol for Isolation of ABP-Induced Mutants by PstI Selection

The *Pst*I selection protocol was the first method used to select for the mutants among the progeny phage from the initial transfection (T_1) of both the ABP-modified and control genomes (Figure 27). Briefly, an aliquot of the progeny phage from T_1 was used to infect fresh M13 host cells, and the RF DNA was isolated from the phage-infected cells. The RF DNA was subjected to restriction by *Pst*I to cleave wild-type DNA, and subsequently transformed into competent *E. coli* MM294A cells. Since the ABP adduct resides in the unique *Pst*I restriction site, 5'-CTGCAG-3', any mutations within this site should inactivate the *Pst*I recognition sequence. The selection protocol was based upon two reasonable assumptions: first, the adduct induced mutants in the *Pst*I site were resistant to cleavage by this enzyme; and second, that linear RF DNA transforms at a very low efficiency (< 1.0% compared to circular RF DNA). The mutants were amplified by a further three rounds of selection and transformation. Single plaques obtained after the fourth round of transformation (T_n) were isolated; their ss DNA was prepared and then sequenced to determine the types of mutations that occurred at the *Pst*I site.

Figure 27.

*Pst*I Enzyme Mutant Selection Protocol. Mutations within the *Pst*I restriction site were selected as described.



Determination of mutant phage among original phage population:

Original phage population after transfection 1 (T1):
mutants plus wild type (blue)
mutants plus nonspecific mutants (light-blue, colorless)

⇩ selection with *Pst* I restriction enzyme
removes *Pst* I sensitive phage (wild type)

Amplification of mutant phage relative to wild-type phage

⇩ repeat selection with *Pst* I

Phage population after transfection n (Tn):
selection and sequence analysis of mutant phage

⇩

Mutation frequency: $\frac{\text{mutant phage after Tn}}{\text{total phage after T1}} \times \frac{\text{clear phage after T1}}{\text{clear phage after Tn}}$

Determination of Mutation Frequency of ABP-Induced Mutants

The mutation frequency (MF) was determined by enumerating the number of mutant phage from the final transformation as determined by DNA sequencing and dividing that value by the number of total phage produced after the initial transformation and multiplying this ratio by a correction factor that normalized for variable transformation efficiency. The following formula was used:

$$MF = (M/seq\ B_n) \times (B_n/C_n) \times (C_1/T_1)$$

where M represents the mutant blue phage obtained after the final transformation and seq B_n is the number of blue plaques from the final transformation that were sequenced; B_n equals the total blue phage after the final transformation; C_n represents the number of colorless plaques in the final transformation; C_1 is the number of colorless plaques observed from the first transfection and T_1 is the total number of progeny phage produced from the initial transfection. The number of colorless plaques was used as an internal standard since this value was not expected to change during the course of the *Pst*I selection protocol.

A typical calculation of MF induced by the site-specifically ABP-modified genome was performed as follows. To obtain the value for the mutation frequency of the M13-TG-(ABP)CA genome replicated in DL7/pGW16 SOS-induced cells (MF presented in Table 6), an average of the induced MF from two independent transfections was taken. To determine the MF the following calculations were made using the mutant selection protocol described in Figure 27. After the final or fourth transformation (T_n or in

this case, T_4), 60 putative mutant ss DNAs were sequenced. Of this number, 24 were determined to be wild-type DNAs that may have escaped the *Pst*I selection protocol. From the remaining 36, 24 were sequenced to be untargeted bp substitutions, insertions or deletions and the remaining 12 were sequenced to be targeted bp substitutions at either position 6245 or 6246 of the *Pst*I site. To determine the targeted mutation frequency, I used the formula above as follows:

MF = 12 (M after T_4)/ 60 (seq B_4) X 2 x 10^6 (B_4)/ 9.5 x 10^7 (C_4) X 1.7 x 10^6 (C_1) / 6.0 x 10^7 (T_1) =

0.2 X 2.1 x 10^{-2} X 2.8 x 10^{-2}, which yields an MF of 0.012% for the M13-TG-(ABP)CA genome replicated in DL7/pGW16 (+SOS) cells (Table 6).

Transfection and Mutagenic Efficiency of the dG-C8-ABP Adduct at the PstI Site in an M13 SS Genome

The results of two independent mutation assays using site-specifically modified M13-TG-(ABP)CA, and the control genome, M13-TGCA, are presented in Table 6. The combined results of both assays indicate that the transfection efficiency of the ABP-adducted genome was not remarkedly lower than the unmodified genome in uvr^+ cells. This survival did not increase in SOS-induced DL7 cells containing pGW16. This observation correlates with the random mutagenesis studies in which neither SOS-induction of host cells nor the presence of pGW16 led to an increase in the survival of globally-ABP-modified M13 ss genomes.

Table 6.

Site-specific Mutagenesis Assays

M13 dG-C8-ABP-modified (MIT) ss genomes

Expt 1:

Cells	M13 genome	Survival	Mutation frequency (%)[a]		
		Transfection Efficiency	Targeted	Untargeted	Total
DL7	M13 TGCA	1.2×10^3/ng	< 0.007%	0.023%	0.023%
	M13TG-(ABP)CA	3.4×10^3/ng	< 0.002%	0.007%	0.007%
DL7/pGW16	M13TGCA:	6.7×10^2/ng	< 0.015%	0.015%	0.015%
+ UV	M13TG-(ABP)CA	1.3×10^3/ng	0.012%	0.024%	0.036%

[a] Mutation frequency = Percent of mutant phage among original progeny phage (T1)

Expt 2:

Cells	M13 genome	Survival	Mutation frequency (%)[a]		
		Transfecton Efficiency	Targeted	Untargeted	Total
DL7	M13 TGCA	100%(7.2×10^2/ng)	n.d.	n.d.	n.d
	M13TG-(ABP)CA	53%(4.2×10^2/ng)	n.d.	n.d.	n.d.
DL7/pGW16	M13TGCA:	100%(3.0×10^3/ng)	< 0.011%	0.126%	0.126%
+ UV	M13TG-(ABP)CA	21%(8.5×10^2/ng)	0.016%	0.240%	0.256%

[a] Mutation frequency = Percent of mutant phage among original progeny phage (T1)
n.d. not determined

Using the *Pst*I selection protocol for isolation of mutants, the total mutation frequency for a singly-modified genome was 0.036% compared to 0.015% for the control genome in SOS-induced DL7/pGW16 cells; however, the targeted mutation frequency in the modified sample was only 0.012% (Table 6). In the second experiment, the targeted mutation frequency of a single ABP lesion was calculated to be 0.016%. This latter value, however, was exceeded by a large number of nontargeted mutations with a mutation frequency of 0.240%. These insertion mutations were also present in the control samples.

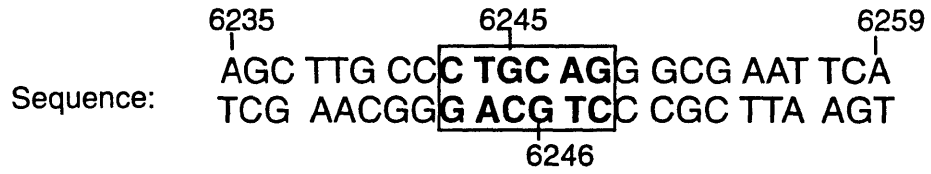
Spectrum of Mutations Induced by the dG-C8-ABP Adduct

Among the mutations analyzed (from Expt 1, Table 6), only targeted mutations were observed in the samples from the transfections with the M13-TG-(ABP)CA genomes. An analysis of the targeted base substitution mutations indicated that they were almost exclusively G -> C transversions that occurred with equal frequency from adducts that could have originated in either the plus strand or the minus strand (Table 7). In these site-specific mutagenesis experiments, targeted mutations were not observed in M13-TG-(ABP)CA genomes replicated in non-SOS-induced cells. The remainder of the mutations were primarily large insertions and deletions, followed by nontargeted base substitutions that occurred within the *Pst*I recognition site. The untargeted mutations were also present in the control samples and probably resulted from the genetic engineering techniques used to construct the genomes.

Table 7.

Mutations observed in Site-specific Mutagenic Assays

M13 dG-C8-ABP modified (MIT) ss genomes



Targeted Mutations:

Cells	M13 genome	Type	Location	Number	MF
DL7/pGW16 +UV	TG-(ABP)CA (MIT)	G -> C	6245	4	0.012%
		G -> C	6246	5	
		G -> T	6245	2	
		G -> T	6246	1	
		G -> A	6245	1	

Untargeted Mutations:

Cells	M13 genome	Type	Location	Number	MF
DL7-UV	TGCA	C -> A	6243	1	0.023%
		insertions	6244-6248	4	
		-21 deletion	6232-6252	2	
DL7-UV	TG-(ABP)CA (MIT)	A -> C	6247	1	0.007%
		insertions	6244-6248	6	
DL7/pGW16 +UV	TGCA	C -> A	6243	1	0.015%
		G -> T	6248	1	
		insertions	6244-6248	6	
DL7/pGW16 +UV	TG-(ABP)CA (MIT)	T -> G	6244	1	0.024%
		T -> C	6244	2	
		A -> G	6247	2	
		-21 deletion	6232-6252	7	
		A -> T	6253		
		insertions	6244-6248	11	

M13 Plaque Hybridization Protocol for Selection of DNA Mutants at the PstI Site

Although the mutagenesis results were obtained using the *Pst*I mutant selection protocol that was successfully employed in earlier studies (Loechler et al., 1984), it was possible that in the current study, the selection protocol used to isolate the mutant phage may have resulted in a biased selection for the nontargeted mutants, specifically the insertion and deletions, which could obscure the real or targeted mutations. Therefore, a differential plaque hybridization technique was used as an additional method for screening the phage population. This procedure involved isolating mutant plaques among wild-type plaques by using radiolabeled probes that would hybridize only to wild-type phage DNA. The positive signal produced would permit the detection of mutant phage, which would be represented as a negative signal when screened with the wild-type probe.

The following tests were performed to confirm that all possible single-base mismatches would be detected in the hybridization analysis of the phage samples. A series of M13 phage DNAs mutated at a single position in the *Pst*I site, 5'-CTGCAG-3,' was used to test the efficacy of the hybridization conditions. Briefly, wild-type phage plaques were stippled onto a 10 x 10 grid on an *E. coli* NR8044 overlayed B Broth plate. Mutant phage samples were then stippled at specific positions among the wild-type phage in the grid. Filters were made of these grids and probed with a 5'-[^{32}P]-ATP labeled 15-mer probe with a sequence that was complementary to the wild-type phage DNA at the *Pst*I restriction site. The hybridization

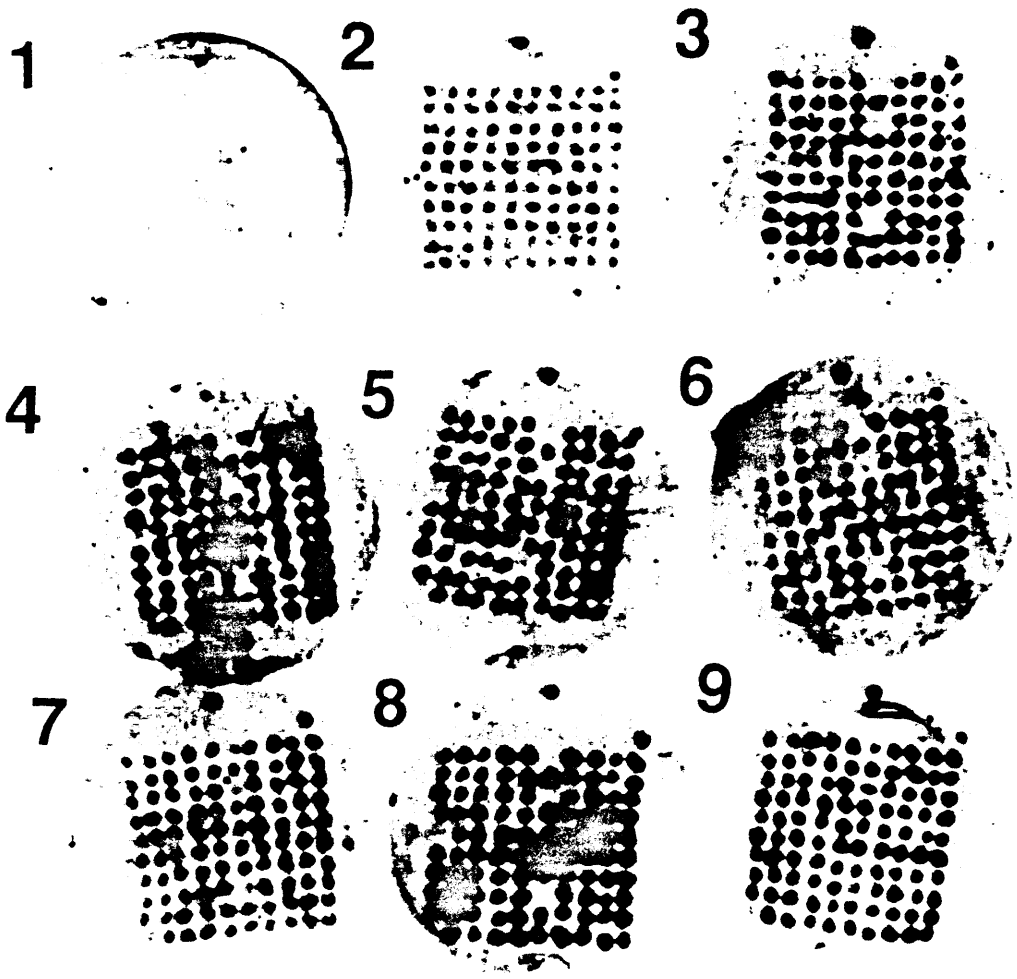
and washing conditions were adjusted so that the wild-type probe hybridized only to wild-type M13 plaque DNA but not to DNA sequences that had a single base mutation (Figure 28).

This technique was used to analyze the phage from the transfection experiments. After plating aliquots of the progeny phage from the first transfection (T_1), phage plaques produced on a plate were transferred by stippling onto a bacterial lawn in a grid. Filters made from the plaque grids were screened with the 5'-[^{32}P]-ATP labeled wild-type probe.

Using the plaque hybridization protocol, phage plaques from the transfection samples from Experiment 1 were screened with the wild-type radiolabeled probe. The probe hybridized only to wild-type M13 plaque DNA but not to DNA sequences that had a single base mutation at the *Pst*I site. In this manner, the putative mutant plaques were detected, subsequently plaque-purified and analyzed by DNA sequencing. Using the hybridization protocol, no mutant plaques were detected in 3 x 10^3 progeny phage plaques produced from the initial transfection (T_1) of M13-TG-(ABP)CA genomes in SOS-induced DL7/pGW16 cells. Subsequently, 3 x 10^3 phage plaques from the progeny phage pool that was selected with *Pst*I and amplified once were analyzed for mutant phage. This analysis yielded 9 mutant plaques among 3000 total plaques. The targeted mutation frequency was calculated to be 0.007% in the progeny phage from the initial transfection, which correlates reasonably well with the value of 0.012% obtained from mutant selection using the *Pst*I enzyme digestion and rounds of selection method *vide supra*. The results of the hybridization analysis confirmed that the *Pst*I protocol was a reasonable method of mutant selection.

Figure 28.

Experiment to establish conditions for the M13 plaque hybridization protocol for mutant selection. M13 plaque grids were made on a B Broth plate that had an overlay of the M13 *opal* suppressor host cells. A 10 x 10 grid of stippled wild type (at the ***Pst***I site) phage was made on the plate. At certain positions within the grid, specifically positions, 5, 25, 50, 75, and 100, phage with known mutations at the ***Pst***I site were stippled to be used as "negative" controls. Filters were then made of the different grids and probed with a radiolabeled wild-type probe. Conditions were adjusted to permit detection of mutant plaques among wild-type plaques. Filter 1: no plaque control; Filter 2: all wild-type plaques; Filter 3: C (-4 base deletion mutants at ***Pst***I site) mutant plaques stippled at specified positions; Filter 4: T5 (G -> T at position 6245) mutant plaques stippled at specified positions; Filter 5: T6 (G -> T at position 6246) mutant plaques; Filter 6: A5 (G -> A at position 6245) mutant plaques; Filter 7: A6 (G -> A at position 6246) mutant plaques; Filter 8: C5 (G -> C at position 6245) mutant plaques; Filter 9: C6 (G -> C at position 6246) mutant plaques.


1
2
3
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6
7
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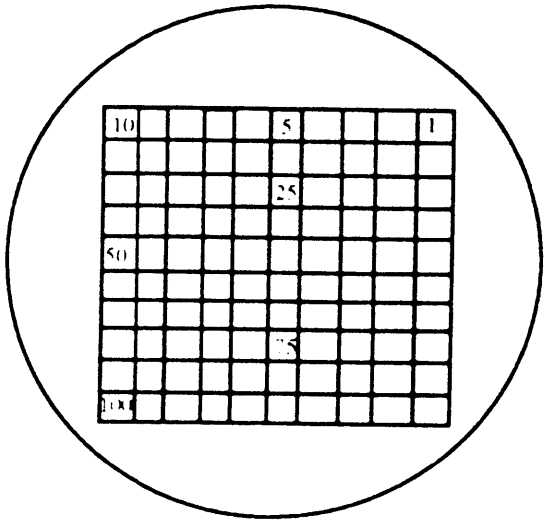

10
5
1
25
50
5
100

Detection of opal Mutations at the PstI Site

One type of expected base substitution mutation could be readily screened in the *lacZα* mutation assay. This is a G:C -> T:A bp substitution in the minus strand at position 6246. This mutation results in a TGA (*opal*) stop codon in the reading frame of the β-galactosidase gene encoded by the *lacZα* fragment of the M13 genome (Figure 29). These mutations were detected as colorless plaques against a background of wild-type blue plaques when the phage were plated on the M13 host *E. coli* GW5100 (an *amber* suppressor strain). The plaques exhibited a light-blue phenotype, however, when plated on an *opal* suppressor strain, *E. coli* NR8044. M13 phage DNA was isolated from these putative mutants and the mutation was confirmed by sequence analysis.

Using the plaque phenotype method of mutant selection, the frequency of the G -> T transversions was determined to be approximately 0.01% for M13-TG-(ABP)CA genomes assayed in Experiment 1. Many of the putative *opal* mutants, which produced the light-blue phenotype, were sequenced to be mutations at sites other than at the *Pst*I site.

To summarize, the results of three independent methods of mutant selection or screening have indicated that the mutation frequency of a single ABP lesion situated at the *Pst*I site in an M13 single-stranded genome is approximately 0.01% in our experimental system.

Figure 29.

Protocol for detection of in-frame *opal* mutants. The normal gene sequence and the mutant gene sequence are depicted.

Normal M13mp19+ sequence

Amino acid sequence:	**Ser**	**Leu**	**Pro**	**Cys**	**Arg**	**Ala**	**Asn**	**Ser**
				6245				
+ strand :	AGC	TTG	CCC	TGC	AGG	GCG	AAT	TCA
- strand:	TCG	AAC	GGG	ACG	TCC	CGC	TTA	AGT
				6246				

Sequence after G -> T transversion at position 6246

Amino acid sequence:	**Ser**	**Leu**	**Pro**	**STOP**				
+ strand :	AGC	TTG	CCC	TGA	AGG	GCG	AAT	TCA
- strand:	TCG	AAC	GGG	ACT	TCC	CGC	TTA	AGT
				6246				

Protocol:

Original phage population after transfection 1 (T1):
mutants plus nonspecific mutants (colorless)

⇩

Screen for mutants by plating on opal suppressor strain:
mutants (light blue)

⇩

Mutation frequency: $\frac{\text{mutant phage after Tn}}{\text{mutant phage after T1}}$ **X** $\frac{\text{clear phage after T1}}{\text{clear phage after Tn}}$

Initial Site-Specific Mutagenesis Studies Conducted with M13 Singly ABP-Adducted (NCTR) Genomes in *E. coli*

This section describes the results from the initial site-specific mutagenesis studies conducted with M13 genomes made with the ABP-adducted tetranucleotide, 5'-TG-(ABP)CA-3' (NCTR). The synthesis and characterization of the NCTR tetranucleotide used in the initial experiments, described herein, was reported by Lasko et al. (1987). Throughout this section, I shall refer to the ABP-tetranucleotide used in these initial experiments as TG-(ABP)CA (NCTR).

A site-specifically modified genome was constructed by ligating either the modified tetranucleotide, 5'-TG-(ABP)CA-3'(NCTR), or the corresponding unmodified tetramer, 5'-TGCA-3' into a unique *Pst*I site restriction enzyme site in a M13 gapped heteroduplex DNA vector. After its construction, the ABP-modified genome was characterized to map the location of the ABP-DNA adduct in the vector. The results of the *Pst*I restriction enzyme digestion assay, used as a criterion to assess the purity of the ABP-modified genome, indicated that the ABP-tetranucleotide may have undergone some degradation since its synthesis at NCTR. Aware of the technical problems, I proceeded to conduct mutagenesis experiments with the ABP-modified genome (NCTR), taking some precautions prior to transfection into cells. The initial mutagenesis results and sequencing data were encouraging and were consistent with the mutational spectrum obtained from globally ABP-modified ss genomes.

Although these initial results were encouraging, I have repeated the site-specific mutagenesis experiments with a freshly-made tetranucleotide,

5'-TG-(ABP)CA-3' (MIT). The results of these experiments conducted with MIT-modified tetranucleotide indicated that the dG-C8-ABP adduct was not significantly mutagenic at the *Pst*I site in the M13 ss genome. The results of the initial site-specific experiment, however, indicated that the dG-C8-ABP was mutagenic, albeit at a low level (9-fold above background), in SOS-induced cells. The analysis of TG-(ABP)CA (NCTR), the characterization of M13 genomes constructed with TG-(ABP)CA (NCTR), and the results from the initial site-specific mutagenesis experiments are described in the following sections.

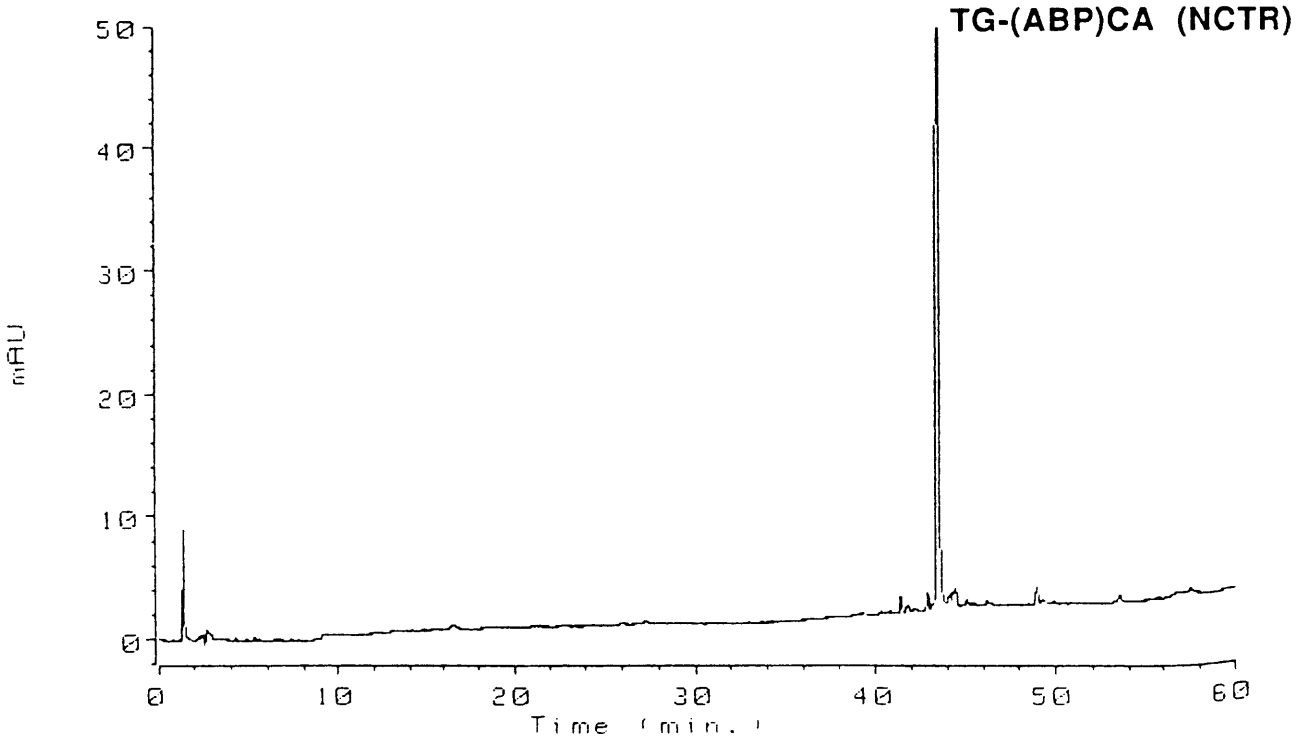
Characterization of the Tetranucleotide, d[pTpG-(ABP)pCpA] (NCTR)

Analysis of the ABP-adducted tetranucleotide, TG-(ABP)CA (NCTR), by nucleoside digestion and HPLC analysis indicated that some contaminants, at a level of ~10%, may be present in the TG-(ABP)CA (NCTR) sample (Figure 30) compared to the analysis of TG-(ABP)CA (MIT) (Figure 21b). In addition, FAB mass analysis of the TG-(ABP)CA (NCTR) oligonucleotide indicated that the $[M - H]^-$ ion was at *m/z* 1340; however, there was also a significant peak at *m/z* 1339. The molecular weight of the TG-(ABP)CA (NCTR) after its initial synthesis at NCTR was inferred to be 1340 from earlier mass spectrometry analysis (Lasko et al., 1987). In comparison, analysis by mass spectrometry of TG-(ABP)CA (MIT) indicated that the major $[M - H]^-$ ion was at *m/z* 1339, which correlates with a accurate molecular weight of 1340 for TG-(ABP)CA (MIT).

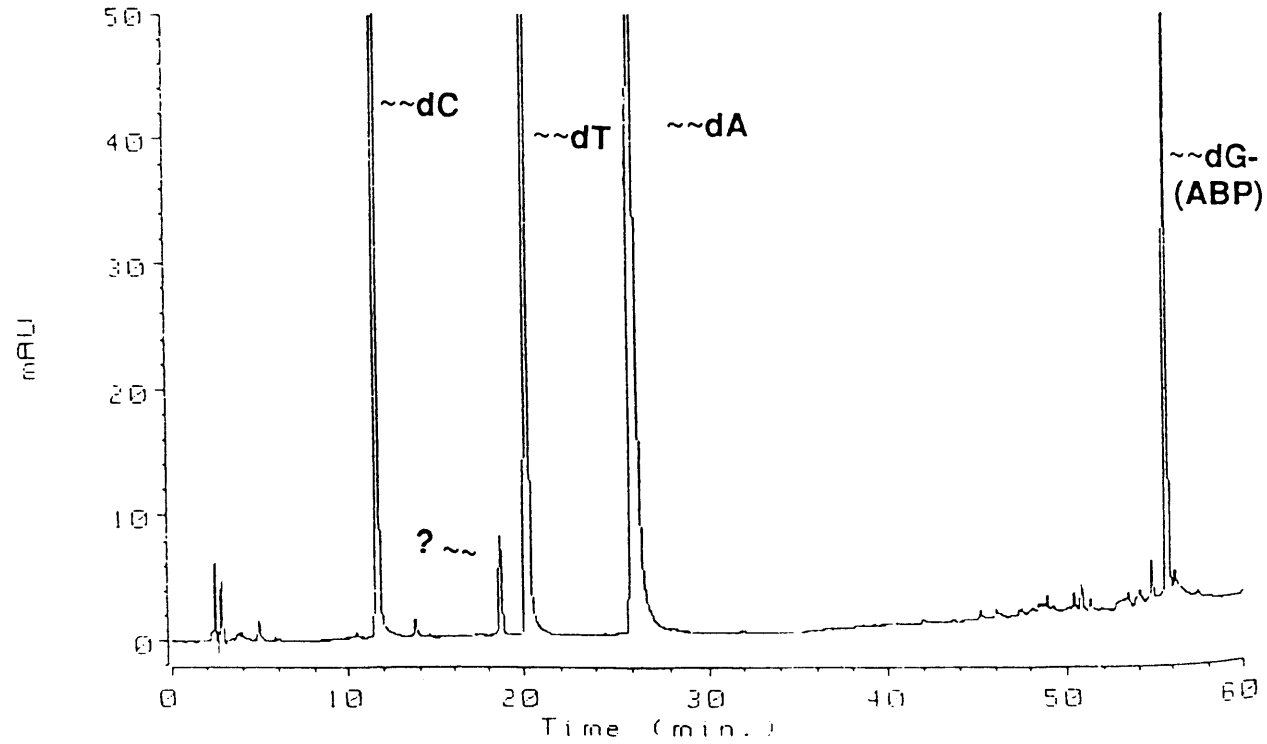
Figure 30.

Analysis of the tetranucleotide, 5'-TG-(ABP)CA-3'. a. HPLC analysis of the tetranucleotide, 5'-TG-(ABP)CA-3' (NCTR). b. Nucleoside composition analysis of TG-(ABP)CA (NCTR) by nuclease P_1 and snake venom phosphodiesterase digestion.

a) TG-(ABP)CA (NCTR)



b) TG-(ABP)CA (NCTR) digestion



Construction and Characterization of Singly-ABP-Modified Genomes

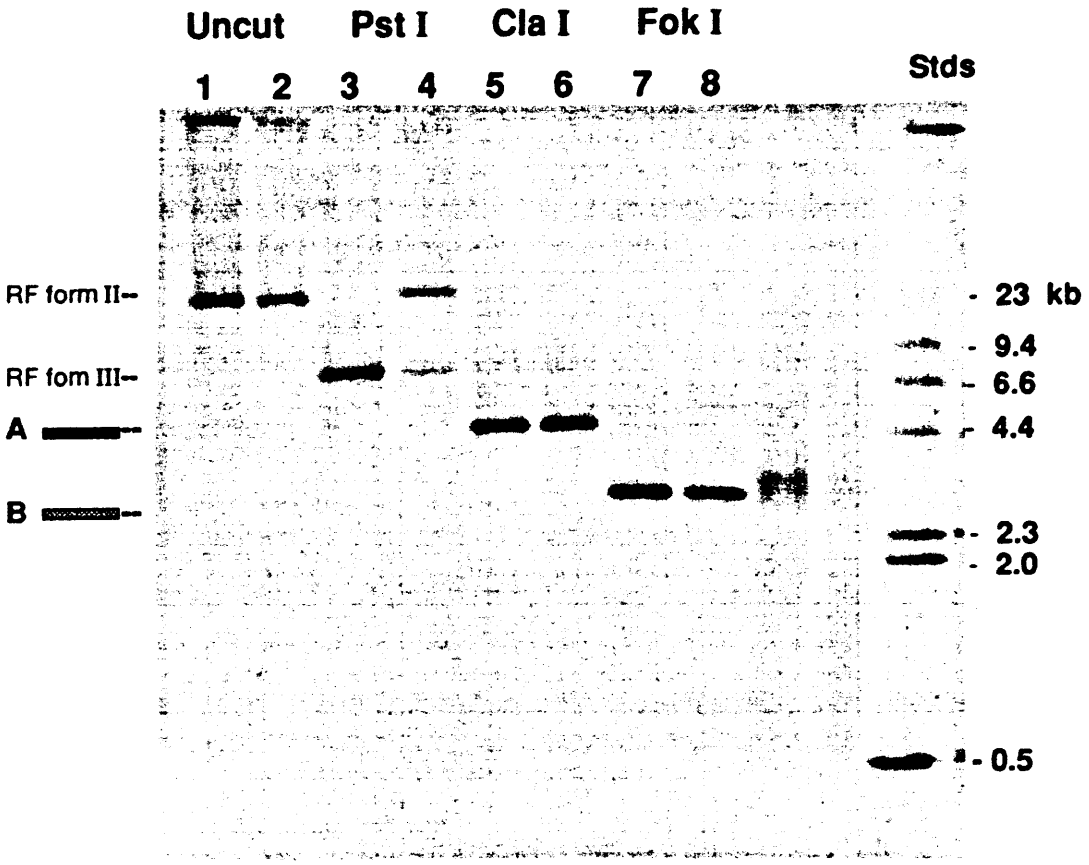
A site-specifically modified genome was constructed by ligating either the modified tetranucleotide, 5'-TG-(ABP)CA-3'(NCTR), or the corresponding unmodified tetramer, 5'-TGCA-3' into a unique *Pst*I site restriction enzyme site in a M13 gapped heteroduplex DNA vector. The site-specifically modified genome, denoted as M13-TG-(ABP)CA (NCTR), together with the control genome, M13-TGCA, were characterized with restriction enzyme digestions followed by gel analysis as described above. The *Pst*I restriction assay was used as a criterion to assess the purity of the genome as well as to confirm the location of the ABP-DNA adduct in the vector. Restriction analysis of the genome, M13-TG-(ABP)CA (NCTR) resulted in a significant fraction of the genomes being sensitive to digestion by *Pst*I (Figure 31). These results are in contrast to the results obtained for the *Pst*I digestion assay for the genome M13-TG-(ABP)CA (MIT) (Figure 24). These earlier results suggested that the ABP-tetranucleotide used for the construction of M13-TG-(ABP)CA (NCTR) may have undergone some degradation since its initial synthesis at NCTR.

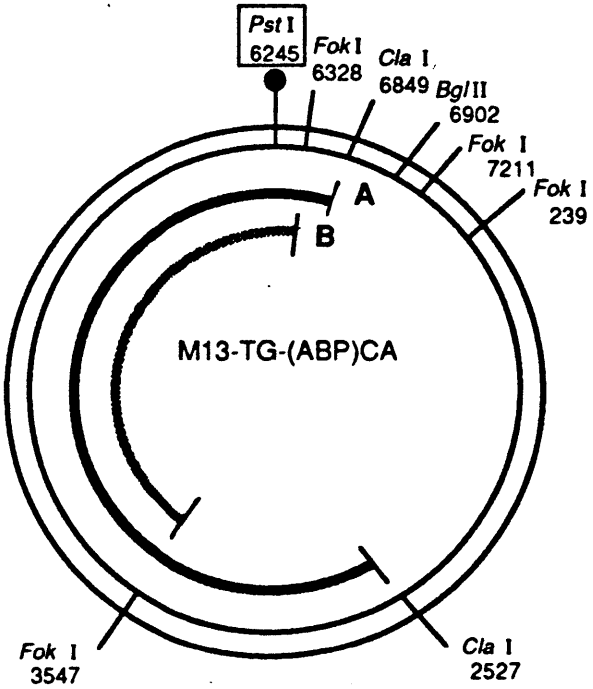
Transfection Assays with the Singly-ABP-Modified Genome

Although the purity of the M13-TG-(ABP)CA (NCTR) genome was in question, I treated this ABP-genome with the *Pst*I enzyme prior to any mutagenesis experiments, reasoning that the treatment would possibly eliminate contaminants present in the sample. The linear DNA generated from the *Pst*I digestion would become biologically inviable when the genomes were heat denatured prior to transfection into *E. coli*.

Figure 31.

Restriction enzyme analysis of the site-specifically modifed genome, M13-TG-(ABP)CA (NCTR), and the control genome, M13-TGCA. The uncut M13TGCA and M13TG-(ABP)CA genomes migrate as RF form II (nicked circular) DNA (Lanes 1 and 2, respectively). Both the ligated M13TGCA and M13TG-(ABP)CA genomes were digested with the enzymes, *Pst*I, *Cla*I, and *Fok*I to map the position of the ABP adduct in the M13TG-(ABP)CA genome and to determine if the *Pst*I digestion was inhibited by the ABP adduct at the *Pst*I site. *Pst*I digestion of the M13TGCA genome linearizes the nicked circular genome, from RF form II DNA, to RF form III DNA (Lane 3). *Pst*I digestion of the ABP-modified genome indicates that a significant proportion of the genomes are linearized by *Pst*I from form II to form III (Lane 4). Digestion of the M13TGCA and the M13TG-(ABP)CA genomes with either *Cla*I (Lanes 5 and 6, respectively) and *Fok*I (Lanes 7 and 8, respectively) yield the expected sized fragments.


Uncut
Pst I
Cla I
Fok I
1 2 3 4 5 6 7 8
Stds
RF form II
RF fom III
A
B
23 kb
9.4
6.6
4.4
2.3
2.0
0.5


Pst I
6245
Fok I
6328
Cla I
6849
Bgl II
6902
Fok I
7211
Fok I
239
A
B
M13-TG-(ABP)CA
Fok I
3547
Cla I
2527

The control genome, M13-TGCA, and the adducted genome, M13-TG-(ABP)CA (NCTR), were denatured to produce ss genomes and then transfected into SOS-induced DL7 (*uvr*$^+$) cells, SOS-induced DL7/pGW16 (*uvr*$^+$/(*mucAB*)) or uninduced DL7 (*uvr*$^+$) cells using the electrotransformation protocol (Figure 14). After transformation, an aliquot of the transfection mixture was plated to determine transfection efficiency of the modified genome and compare it to the value obtained for the control genome. Subsequently, progeny phage produced from the initial transfection (T_1) were plated and enumerated as the first step in determining mutation frequency.

Mutation Frequency of the Singly ABP-Modified Genome

To isolate mutant phage, rounds of mutant selection were performed to amplify mutant phage with respect to wild-type phage with the *Pst*I selection protocol as detailed above (Figure 27). Transfection efficiency and mutation frequency of M13-TG-(ABP)CA (NCTR) compared to M13-TGCA were determined.

The results of one site-specific mutagenesis experiment are presented in Table 8. In this study, a decrease in transfection efficiency was observed between ABP-modified genomes and control genomes; however, the survival of ABP-modified genomes did not increase with SOS-induction of DL7 or DL7/pGW16 host cells. The mutagenesis data from this experiment indicate that the dG-C8-ABP was mutagenic, albeit at a low level (9-fold above background), in SOS-induced DL7/pGW16 cells. The average adduct-induced mutation frequency was determined to be 0.09% for the M13-TG-(ABP)CA (NCTR) genome replicated in these cells.

Table 8.

Site-specific Mutagenesis Assays

M13 dG-C8-ABP-modified (NCTR) ss genomes

Cells	M13 genome	Survival	Mutation Frequency(%)[a]		
		Transfection Efficiency	Targeted	Untargeted	Total
DL7	M13 TGCA	100% (2.8 x 10^4/ng)	<0.01%	0.06%	0.06%
	M13TG-(ABP)CA	53% (1.5 x 10^4/ng)	0.01%	0.01%	0.02%
DL7 +UV	M13TGCA	100% (2.8 x 10^3/ng)	< 0.02%	0.02%	0.02%
	M13TG-(ABP)CA	43% (1.2 x 10^3/ng)	0.06%	<0.01%	0.06%
DL7/pGW16 +UV	M13TGCA	100% (1.3 x 10^4/ng)	<0.01%	0.05%	0.05%
	M13TG-(ABP)CA	21% (2.8 x 10^3/ng)	0.09%	0.05%	0.14%

[a] Mutation frequency= percent of mutant phage among original progeny phage after T1

Two conclusions were made based on these results: first, survival of the ABP-adducted genome was not enhanced by SOS-induction of host cells; and second, the ABP-modified genome exhibited a higher mutation frequency in DL7/pGW16 cells compared to DL7 cells. The results are consistent with the survival of globally ABP-adducted ss genomes in *E. coli* (Figure 17 and Table 4). The mutagenesis results also suggest that the *mucAB* mutagenic genes, which are present on plasmid pGW16, are required to observe high mutation frequencies with ABP-DNA adducts, especially in ss DNA.

As an alternative selection protocol, the progeny phage from the first transfection (T_1) of this experiment were analyzed by the hybridization screening protocol. Using this method of analysis, a targeted mutation frequency of 0.07% was determined; this result concurred with the mutation frequency of 0.09% obtained by the *Pst*I selection protocol described above. The results of the hybridization analysis confirmed that the *Pst*I protocol was a reasonable method of mutant selection.

Spectrum of Mutations Induced by the dG-C8-ABP Lesion at the PstI Site

An analysis of the mutations induced by the ABP lesion in ss DNA revealed that they were almost exclusively G -> T and G -> A changes targeted to site of the adduct (Table 9). These data are consistent with the mutation spectrum from globally-modified ss genomes in which the primary mutation was the G -> T transversion. A large number of untargeted mutations were observed in the SOS-induced DL7/pGW16 cells; some of these, especially the -21 deletion, which has been observed in the recent experiments (Table 6), may be due to the nature of the sequence context at

Table 9.

Mutations observed in Site-specific Mutagenic Assays

M13 dG-C8-ABP-modified (NCTR) ss genomes

6232 6245 6259

Sequence: ACG CCA AGC TTG CC**C TGC AG**G GCG AAT TCA
TGC GGT TCG AACGG**G ACG TC**C CGC TTA AGT

6246

Targeted Mutations:

Cells	M13 genome	Type	Location	Number	MF[a]
DL7 -UV	TG-(ABP)CA (NCTR)	G -> T	6245	2	0.01%
		G -> A	6246	1	
DL7 +UV	TG-(ABP)CA (NCTR)	G -> T	6245	6	0.06%
		G -> A	6246	1	
DL7/pGW16 +UV	TG-(ABP)CA (NCTR)	G -> T	6245	5	0.09%
		G -> A	6245	7	
		G -> A	6246	2	
		G -> C	6245	2	

[a] Mutation frequencies are from Table 8. (% mutant phage among T1)

Untargeted Mutations:

Cells	M13 genome	Type	Location	Number	MF[a]
DL7-UV	TGCA	-21 deletion	6232-6252	2	0.06%
		G -> T	6248	2	
	TG-(ABP)CA	-21 deletion	6232-6252	2	0.01%
DL7/pGW16 +UV	TGCA	-21 deletion	6232-6252	5	0.05%
		T -> A	6244	4	
	TG-(ABP)CA	T -> A	6244	8	0.05%
		A -> T	6247	1	
		A -> G	6247	1	

[a] Mutation frequencies are from Table 8. (% mutant phage among T1)

the *Pst*I site. The untargeted bp substitution mutations which appeared in both the control and the ABP-modified samples probably were the result of genetic engineering techniques used to construct the genomes.

The mutational changes observed in this experiment were consistent with the ABP mutational spectrum, but they were not consistent with the mutations induced by the genomes made with the chemically-pure TG-(ABP)CA (MIT). Some contaminants, present at a level of ~10%, were observed in the HPLC analysis of the NCTR ABP-modified tetranucleotide (Figure 30). It is possible, but not certain, that these contaminants or putative breakdown products of TG-(ABPCA (NCTR) may be the components responsible for the different types of mutations in my initial experiments with the M13-TG-(ABP)CA (NCTR) genomes. As stated above, in studies conducted with genomes made with the newly synthesized, pure MIT ABP-adducted tetramer, I observed far less mutagenesis and, importantly, a different mutation specificity for the dG-C8-ABP adduct.

V. DISCUSSION

In this section, I shall discuss the results from both my *lac*Z gene random ABP mutagenesis studies and the site-specific dG-C8-ABP mutagenesis experiments. I shall also relate my results to previous studies investigating mutagenesis induced by ABP and related aromatic amines such as AF.

The results of an earlier study conducted in our laboratory had indicated that a single dG-C8-ABP adduct when present in only one strand of a ds M13 genome causes a low or undetectable mutation frequency in *E. coli* (Lasko et al., 1988). A bias against replication of the ABP-adducted strand of the singly-modified vector was suggested as one explanation for this observation. In addition, other studies carried out in our laboratory had shown that a single chemical-DNA adduct, including O^6-methylguanine, thymine glycol, and the major G^G adduct of *cis*-platin, when present in ss DNA was more mutagenic than the same lesion situated in ds DNA (Essigmann et al., 1986; Basu et al., 1988; Bradley, 1991).

To address the question of whether a single ABP-DNA adduct in M13 ss DNA could induce mutations, three studies were undertaken. First, the UV genotoxicity assays were carried out to be used as a model system in *E. coli* cells in which to study the toxicity and the mutagenesis by DNA adducts of ABP; second, the *lac*Z gene random mutagenesis assays were performed to determine which of the known DNA adducts of ABP were mutagenic in the M13 ss genome and to compare the mutational spectrum in ss DNA with the results previously observed in ds DNA (Lasko et al., 1988); and third,

the site-specific mutagenesis experiments were conducted to determine the genotoxic and mutagenic effects of the major ABP-DNA adduct in M13 ss DNA.

UV Genotoxicity Assays

The results from the preliminary UV assays provided information about the toxicity of bulky or helix-distorting DNA lesions such as those formed by UV radiation in bacterial strains of different repair and mutagenic backgrounds. The combined results from the UV assays indicated that the *uvr* excision repair system protects the host cell from the damaging effects of UV irradiation and the *muc*AB gene products enhance mutagenesis induced by UV lesions in ss DNA. The results also correlate with the previous results obtained with UV-irradiated M13 ss DNA replicated in uvr^+ and uvr^- cells (Lasko, 1986). From these assays it was demonstrated that DL7/pGW16 (uvr^-/*mucAB*)) induced for SOS functions were the optimum cells in which to study mutagenesis induced by single ABP lesions.

Mutagenesis Studies in the *lacZ* Gene of Globally ABP-Modified M13 Genomes

The results from the random mutagenesis experiments demonstrate that ABP lesions are both genotoxic and mutagenic in M13 bacteriophage genomes replicated in *E. coli*. ABP adducts in M13 ss DNA are 50-fold more genotoxic than in M13 ds DNA (Figure 17). In contrast to the results found with ds DNA, excision repair proficiency of the host cell did not enhance the survival of ABP-modified ss DNA. Hence, ABP lesions in ds DNA are likely removed by the excision repair enzymes of the host cell but the same lesions in ss DNA apparently cannot be effectively repaired. My

results additionally indicate that SOS-induction of the host cells leads to an increase in the survival of ABP-modified RF DNA (Figure 16). A large number of ABP lesions (e.g. 93, adducts/genome) can be lethal to the M13 genome if the host enzymes cannot repair the lesions. In addition, the persistence of these unrepaired adducts can result in mutations when the DNA is replicated. This was evidenced in the high toxicity and the increase in mutation frequency of ABP lesions observed in *uvr*A cells.

The data from assays with both ABP-modified ss and ds genomes indicate a dose-dependence of the number of ABP adducts per genome with an increase in mutation frequency in both SOS-induced *uvr*$^+$ and *uvr*A cells. SOS-induction of host cells substantially increases the mutagenic impact of ABP lesions in M13 ds DNA (Figure 18). In assays with M13 ss DNA, ABP lesions were found to be more mutagenic when the host cells contained the plasmid pGW16, which carries the *mucAB* mutagenic processing genes (Figure 19).

The mutation frequency of the average ABP adduct in the *lac*Z gene of duplex RF DNA was determined to be 0.09% (calculated in RESULTS chapter) in SOS-induced DL7/pGW16 cells whereas the mutagenic efficiency of the average ABP lesion in the same mutational target of ss DNA was calculated to be approximately 6.2% (see RESULTS chapter) in SOS-induced DL7/pGW16 cells. These results clearly indicate that adducts in ss DNA are approximately 70-fold more mutagenic than in ds DNA, underscoring the importance of an unmodified DNA strand complementary to the adducted strand; the former serves as a template for repair.

The mutagenesis results obtained with the M13 ABP-modified RF genomes correlate with previous results obtained in our laboratory with randomly

ABP-modified ds genomes replicated in SOS-induced cells as reported in our earlier study (Lasko et al., 1988). The value of 0.09% for the mutagenic efficiency of a single ABP lesion in the *lac*Z gene target of an RF genome with 93 adducts in my studies correlates with the mutation frequency of 0.14% obtained for average ABP lesion in an RF genome containing 140 ABP adducts (Lasko et al., 1988).

It was possible to estimate the number of abasic sites formed *in vitro* in the ABP-modified DNA and, on the basis of this analysis, I determined that the mutagenesis observed was predominantly due to the ABP lesions. It is possible, however, that AP sites could have formed inside the cell through some as yet unknown ABP repair system. These intracellularly formed AP sites may have contributed to the increase in mutagenesis observed in my experiments. It was important to perform the AP site analysis, because the predominant mutation observed for ABP lesions (the G -> T transversion) matches the primary mutation from AP sites. Hence, it was essential to rule out the possibility that our observation was an artifact of chemical depurination of ABP-adducted DNA. The results of previous analysis of ABP-modified DNA also demonstrated that AP sites were not formed in significant levels after reaction of activated ABP compounds with M13 DNA (Lasko et al., 1988).

The results presented in this dissertation also correlate with another mutagenesis study with the structurally related carcinogen, AF (Gupta et al., 1988). This study investigated the genotoxic and mutagenic effects of AF lesions in M13 ss and ds DNA. The results from this study also suggest

that AF adducts in ss DNA were more toxic and more mutagenic than adducts in ds DNA. A mutation frequency of 2.5×10^{-3} in SOS-induced cells was observed for an M13 ss genome containing 5 AF adducts. This correlates with my studies in which I observed a mutation frequency of 4.2×10^{-3} for M13 ss genome with 5 ABP adducts replicated in SOS-induced cells (Table 4). These investigators also reported mutagenesis results for AF-modified M13 RF DNA that were similar to the results that I had obtained for ABP-modified M13 RF DNA.

The mutation data also agree with studies conducted in the laboratory of Fuchs and coworkers with plasmid DNA modified with *N*-OH-AF (Bichara and Fuchs, 1985). This study demonstrated that AF-DNA adducts were significantly mutagenic in plasmid DNA only under SOS-induction of host cells. It appears, therefore, that the much studied carcinogen, AF, which differs from ABP only by a methylene bridge, is probably a good model for ABP mutagenesis. The toxicological importance of this conclusion stems from the fact that AF is a compound of relatively minor human health significance, as compared to ABP, but there is a wealth of information in the literature on its biological effects.

The mutation data indicate that ABP is a versatile mutagen, inducing a variety of mutational changes in both SOS-induced and uninduced cells. In uninduced (*uvr*$^+$) cells, ABP induces both G -> T and G -> C transversions. In SOS-induced cells, however, the G -> T transversion was the predominant mutation observed (Table 5). The mutation data suggest that both the dG adducts of ABP, dG-C8-ABP and dG-N^2-ABP, could have contributed to the mutational spectrum. The mutational spectrum for ABP in the *lac*Z gene of

M13 ss DNA was similar to the mutational spectrum for ABP in ds DNA as established in our labortory by Lasko et al. (1988). The predominant changes in the *lacZ* gene of M13 ds DNA were G -> T and G -> A substitutions.

The mutation data also correlated with studies conducted by Gupta et al. (1988), in which AF was found to be a versatile mutagen; the mutational changes induced by AF included all three possible bp substitutions at putative dG-C8-AF adducts. The results also agree with the results obtained in AF-modified plasmid DNA, in which the primary mutation observed was the G -> T transversion (Bichara and Fuchs, 1985). Since *N*-OH-AF predominantly forms the dG-C8-AF adduct after reaction with DNA, it can be speculated that that the mutational change caused by the dG-C8-AF lesion is the G -> T transversion. Site-specific mutagenesis studies with the dG-C8-AF adduct in M13 ds DNA have not entirely resolved this speculation but recent results indicate that this adduct induces a G -> T mutation when situated in an M13 duplex genome containing uracil residues in the strand opposite that in which the dG-C8-AF adduct was situated (Reid et al., 1990).

The mutagenesis results from my experiments conducted with ABP-modified ss DNA provide strong evidence that adducts in ss DNA were more toxic and more mutagenic than adducts in ds DNA. From the results of both the UV genotoxicity and the random ABP mutagenesis assays, it appears that the DL7/pGW16, (*uvr*$^-$/*muc*AB) cells induced for SOS functions are the optimum cells in which to study mutagenesis induced by single ABP lesions. The

mutation data and the correlation of my work with previous results suggest that perhaps the major dG-C8-ABP adduct formed by ABP is responsible for the majority of the ABP-induced mutations in the mutational spectrum.

Site-Specific Mutagenesis Studies by the Major DNA Adduct of ABP

The results of the random mutagenesis studies confirmed that the M13 ss genome would be the most useful vector in which to study site-specific mutagenesis by the major DNA adduct of ABP, dG-C8-ABP. Subsequent to obtaining the mutational spectrum for ABP in M13 ss DNA, a site-specifically ABP-modified genome was constructed and replicated *in vivo* to study mutagenesis induced by a single ABP lesion in the same cells used to conduct the random mutagenesis experiments.

The findings from my site-specific mutagenesis experiments are summarized as follows. Three independent methods of mutant selection/screening have indicated that the targeted mutation frequency of a single dG-C8-ABP lesion in an M13 ss genome is approximately 0.01% in the experimental system used to assay this genome (Table 6). The results of these site-specific mutagenesis experiments have clearly shown that the dG-C8-ABP adduct was not very mutagenic, if indeed it is mutagenic at all, under the conditions of my experiment (adduct located in the *Pst*I site in the M13 ss genome). The low level of mutagenesis observed in these studies was surprising considering that the mutagenic efficiency of a single ABP lesion in the *lacZ* gene of M13 ss DNA was calculated from the random mutagenesis experiments to be approximately 6.2% (in RESULTS chapter).

Analysis of the targeted mutations in the site-specific mutagenesis Experiment 1 was complicated by the low frequency at which ABP induces mutations (Table 6). This made the mutants difficult to detect against the unavoidable background of nontargeted or genetic engineering induced mutations and made it necessary for me to develop, during the course of this work, a mutation analysis system that is an order of magnitude more sensitive than any previously used in our laboratory. In Experiment 2, the frequency of untargeted mutations, specifically the large deletions and insertions, exceeded the frequency of the targeted mutations. It was first believed that the large sequence alterations were the result of the selection protocol used to isolate mutants; however, when the progeny phage from the second round of transformation after one *Pst*I selection were screened for mutants by the plaque hybridization protocol, it appeared that the large insertion and deletion mutants were already present in the phage population. The frequent large deletion mutation (-21) that I have observed throughout these experiments may be due to the nature of the sequence of the genome itself and some hairpin structure formation that becomes deleted when the genomes are replicated *in vivo*.

There may be at least two explanations for the low level of mutagenesis observed in the site-specific mutagenesis experiments; first, the major dG-C8-ABP adduct may not be responsible for the mutagenesis observed in the mutational spectrum studies; and second, the dG-C8-ABP adduct may have been situated in a site not susceptible to ABP-induced mutagenesis (i.e., not a mutational hot-spot sequence). For a sequence to be considered a mutation-

prone sequence, the presence of an adduct at this site may induce a change in the conformation of the DNA such that the lesion becomes a target for the proteins involved in the processing of that lesion into a mutation (Bichara and Fuchs, 1985). If the site were not considered to be a hot-spot sequence, then it is possible that bypass of the ABP lesion *in vivo* may have resulted in the low level of mutations observed in these experiments. Recent studies have demonstrated that the dG-C8-AF adduct when situated in a random DNA sequence was bypassed *in vivo* with accurate translesion synthesis by DNA polymerase I (Michaels et al., 1991). A combination of *in vitro* replication assays and *in vivo* mutagenesis studies with DNA adducts of ABP situated in hot-spot sequences, as deduced from the mutational spectrum, may provide insights into the effect of DNA sequence context on the mutagenic processing of ABP-DNA lesions.

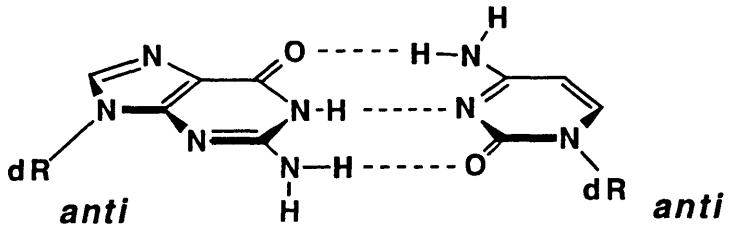
The major mutational change induced, albeit at a low frequency, by the dG-C8-ABP adduct at the *Pst*I site in SOS-induced cells, was the G -> C transversion (Table 7). This bp mutation represented less than 20% of the changes observed in the mutational spectrum for ABP in SOS-induced cells. Although the predominant mutation observed in the *lac*Z mutational spectrum was the G -> T transversion, and not the G -> C transversion, it is possible that at the *Pst*I site, the dG-C8-ABP induces a G -> C mutational change. The mutagenic processing of ABP-DNA adducts in different sequences might result in different mutagenic consequences (E. Loechler, manuscript in preparation).

Studies conducted by Reid et al. (1990) have shown that in SOS-induced cells, the dG-C8-AF adduct induces predominantly G -> T transversions when situated in a duplex genome with uracil residues in the strand opposite that containing the adduct. The mutational spectrum for *N*-OH-AF (a compound that predominantly forms the dG-C8-AF adduct), revealed that AF induces G -> T, G -> C and G -> A mutations with similar frequencies in the *lacZ* gene of AF-modified genomes replicated in SOS-induced host cells (Gupta et al., 1988).

Molecular mechanisms giving rise to the types of mutation induced by ABP have been suggested in an earlier report from our laboratory (Lasko et al., 1988). It is possible that at the *Pst*I site, 5'-CTGCAG-3', the dG-C8-ABP adduct induces a syn conformation about the *N*-glycosidic bond in DNA; in this local architecture, the adducted base may mispair with dG and hence lead to G -> C mutations. Alternatively, in other DNA sequences, the dG-C8-ABP adduct in a syn conformation could mispair with the imino form of dA, which could result in the G -> T transversion (Figure 32). Verification of these models awaits the development of synthetic procedures that will permit preparation of sufficient amounts of ABP-adducted oligonucleotides for analysis by the appropriate spectroscopic techniques.

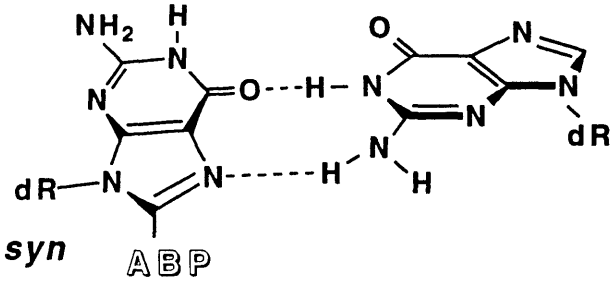
Figure 32.

Biochemical models for hypothesized mechanisms for mutagenesis by ABP-DNA adducts. The normal G:C base-pair is depicted in the top panel. Mechanisms by which G -> C and G -> T mutations could arise from dG-C8-ABP lesions are shown in the middle and lower panels, respectively.



Normal G : C pair

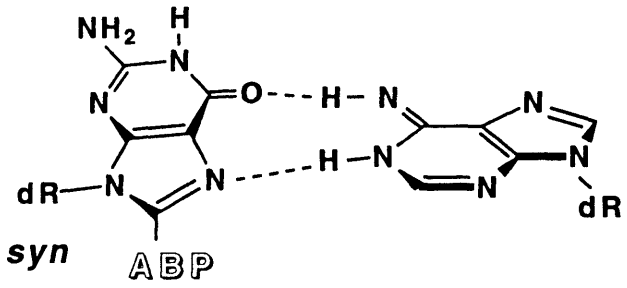
Model to explain G -> C mutations induced by dG-C8-ABP



$_{syn}$dG-C8-ABP : G pair

G -> C mutation

Model to explain G -> T mutations induced by dG-C8-ABP



$_{syn}$dG-C8-ABP : A_{imino}

G -> T mutation

VI. SUMMARY AND CONCLUSIONS

The objective of my research was to determine if the major DNA adduct of ABP, dG-C8-ABP, could cause mutations when situated in the single-stranded M13 bacteriophage genome replicated in *E. coli*.

To address the question of whether ABP lesions in M13 ss DNA could induce mutations, two studies were undertaken. In the first study, ABP mutagenesis assays in the *lacZ* gene of globally ABP-modified M13 DNA were performed to determine which of the known DNA adducts of ABP were likely to be mutagenic in the M13 ss genome and to compare the mutational spectrum in ss DNA with the results observed in our laboratory with ABP-modified ds DNA. In the second study, site-specific mutagenesis experiments were conducted with an M13 ss genome containing a single dG-C8-ABP to determine the genotoxic and mutagenic effects of the major ABP-DNA adduct in M13 ss DNA.

Forward mutation assays were conducted with ABP-modified M13 ss and RF genomes in SOS-induced and uninduced *E. coli* to assess repair and mutagenesis induced by ABP. The results indicated that ABP-DNA adducts are 50-fold more genotoxic and 70-fold more mutagenic in ss DNA than in RF (ds) DNA. Approximately 2 ABP-DNA lesions per ss genome reduced genome survival to 37%, representing a lethal hit, whereas in ds DNA, 37% survival of the genome was attained at approximately 100 adducts/ds genome. The mutation frequency of an "average" ABP-DNA lesion in the *lacZ* gene of ss DNA was determined to be approximately 6.2%, whereas the mutation frequency of a single ABP-DNA adduct in the *lacZ* gene of a ds genome was calculated to be 0.09%.

A partial mutational spectrum for ABP in the *lacZ* gene of ss DNA was determined. ABP lesions in ss DNA induced a variety of mutations at putative deoxyguanosine adducts. Over 50% of the mutations observed in ss DNA were found in two regions of the *lacZ* gene. The dG in the sequence 5'-TGA-3', was found to be the site for mutation for approximately 25% (6/26) of the observed base changes. The spectrum of mutations in ss DNA included G -> T, G -> A, and G -> C bp substitutions; these changes represented 80% of the mutations found in ss DNA.

A deoxytetranucleotide containing a single dG-C8-ABP adduct was synthesized, characterized and incorporated into an M13 single-stranded genome at a unique *Pst*I restriction site. The site-specifically ABP-modified was characterized to confirm the position of the ABP lesion within the genome. The ABP-modified genome and a control unmodified genome were then replicated, in parallel, in SOS-induced and uninduced cells. Mutants were selected and isolated to determine the mutation frequency and the mutational specificity of the major ABP-DNA adduct. To summarize the findings from the site-specific mutagenesis experiments, the results of three methods of mutant selection have indicated that the targeted mutation frequency of a single dG-C8-ABP lesion, situated at the *Pst*I site in an M13 ss genome, was approximately 0.01%.

The major mutational change observed at the site of the adduct was the G -> C transversion. This mutation occurred with equal frequency from adducts that were positioned in either the plus or the minus strand of the genome. From the mutational spectrum of ABP in the *lacZ* gene, the G -> C mutation represented approximately 20% of the bp changes in ss DNA.

VII. SUGGESTIONS FOR FUTURE RESEARCH

My recommendations for future studies are based upon the results presented in this study which, investigated the mutagenesis and the repair of DNA adducts of ABP in *E. coli*.

Further research in this area should investigate the mutagenic effects of the three DNA adducts of ABP situated in different DNA sequence contexts. These investigations can be accomplished by two methods to assess which sequences are mutation-prone sequences for ABP. The first method would utilize *in vitro* DNA replication assays with *E. coli* DNA polymerases on a series of DNA templates containing single ABP-DNA adducts to determine which ABP-DNA adducts are a block to DNA replication and which adducts are bypassed during *in vitro* synthesis. The results from these assays could also determine the effect of DNA sequence on the mutations induced *in vitro* by ABP-DNA lesions as well as determine the frequency of *in vitro* bypass of an ABP lesion in a particular sequence. In addition, by comparing the two dG adducts of ABP, dG-C8-ABP and dG-N^2-ABP, in the same assay system, it may be possible to determine which of these lesions is responsible for the mutational specificity of ABP. The *in vitro* assays would provide some information on the effect of DNA sequence on mutagenesis, which would be valuable prior to embarking on *in vivo* site-specific mutagenesis experiments.

The second method could investigate the *in vivo* mutagenic effects of the major dG-C8-ABP adduct or the minor dG-N^2-ABP adduct in a hot-spot

sequence as deduced from the mutational spectrum. One site that was found to be a hotspot for mutagenesis by putative dG adducts in both ss and ds DNA was the sequence 5'-CGT GAC TGG GAA-3' at position 6307-6318 in the *lac*Z gene. In ss DNA, 11 out of 26 mutations occurred at this sequence and 4 out 14 mutational changes at G:C bps occurred at this site in ds DNA. By testing the individual dG adducts of ABP in this sequence, it may be possible to determine which of the two dG-ABP lesions was responsible for the mutations observed in this sequence.

In addition, it is important to investigate the mutagenic specificity of the minor dA-C8-ABP lesion and assess the contribution of this adduct to the mutational spectrum. Previous studies conducted in our laboratory have revealed a hotspot sequence, 5'-GGGGGA-3', for ABP-mutagenesis in the mutational spectrum at a putative adenine lesion. It would be interesting to conduct site-specific mutagenesis studies investigating the mutagenic specficity of this lesion compared to the major deoxyguanosine adduct in the same experimental system.

Once ABP studies in *E. coli* have been accomplished, the next logical step would be to study mutagenesis by ABP and related compounds in mammalian cell culture assays. ABP is known to be a human bladder carcinogen, and the presence of ABP in our environment has been documented; thus exposure to ABP could have significant implications for many indivduals, particularly smokers. In addition, the related heterocyclic amines, such as the imidazo-quinolines, are known mutagens present in cooked and fried foods. These compounds should also be investigated in both bacterial and mammalian systems.

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BIOGRAPHICAL NOTE

I was born Susan Bina Malaikal on June 16, 1960 in Kerala, India, the daughter of Matthew and Annie Malaikal. I emigrated from India to the US when I was 8 yrs old with my parents and my older brother, Matthew. After attending secondary schools in Rhode Island, in 1978 I joined Tufts University in Medford, MA where I was graduated with a B.S. in Biology in May 1982. After graduation, I worked for two years at Integrated Genetics in Framingham, MA. I entered the former department of Nutrition and Food Science in September, 1984 in the area of Nutritional Biochemistry and Metabolism. In June 1985, I joined the laboratory of my thesis advisor, John M. Essigmann, as a student in the former department of Applied Biological Sciences. I completed my research in Dr. Essigmann's laboratory in the Division of Toxicology in the Whitaker College of Health Sciences and Technology at MIT in February, 1992. In September 1987, I was married to Mammen George Verghis and in March 1989 a daughter, Rachel Nina Verghis, was born to us.