This is to certify that the

thesis entitled

Mutations induced by 4-aminobiphenyl DNA adducts during replication in human cells: Evidence of N-(deoxyadenosin-8-yl)-4-aminobiphenyl as a premutational lesion

presented by

Lucy Xiaotang Wang

has been accepted towards fulfillment
of the requirements for

Master's degree in Medical Technology

Veronica M. Maher
Major professor

Date March 26, 1993

MUTATIONS INDUCED BY 4-AMINOBIPHENYL DNA ADDUCTS DURING REPLICATION IN HUMAN CELLS: EVIDENCE OF N-(DEOXYADENOSIN-8-YL)-4-AMINOBIPHENYL AS A PREMUTATIONAL LESION

b y

Lucy Xiaotang Wang

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Medical Technology Program

1993

ABSTRACT

MUTATIONS INDUCED BY 4-AMINOBIPHENYL DNA ADDUCTS DURING REPLICATION IN HUMAN CELLS: EVIDENCE OF N-(DEOXYADENOSIN-8-YL)-4-AMINOBIPHENYL AS A PREMUTATIONAL LESION

by

Lucy Xiaotang Wang

The mutagen action of a chemical carcinogen, 4-aminobiphenyl, was examined as part of a study to investigate the mechanisms by which carcinogens induces mutations. N-AcO-TFA-ABP, a reactive form of 4-aminobiphenyl, formed a major DNA adduct, dG-C8-ABP. A human embryonic kidney cell line, 293, was transfected with a N-AcO-TFA-ABP-treated shuttle vector. The shuttle vector, pS189, carried the *supF* gene as the target for mutations. A 48-hour period was then allowed for replication of the plasmid. The frequency of *supF* mutants was found to increase with the number of dG-C8-ABP adducts per plasmid. Sequence analysis of the *supF* gene showed that G·C base pairs were the major targets for base substitution (85%), predominantly G·C->T·A transversions (64%). However, mutations were also detected at certain A·T base pairs (15%), and the majority (82%) of them were A·T->T·A transversions. 13% of the mutants exhibited frameshift mutations. This study suggests that the minor dA-C8-ABP adduct plays a role in the specificity of mutagenesis by 4-aminobiphenyl.

ACKNOWLEDGMENT

First of all, I would like to express my deep appreciation to my major professor, Dr. Veronica M. Maher, who helped me not only as a truly dedicated advisor in my research, but also as a very concerned and sincere friend in my personal life. Without Dr. Maher's guidance, encouragement, and consistent concern, this work could not have been accomplished.

The deepest appreciation and love go to my husband, Yong Zhang, for his constant support and understanding through the duration of this work. Yong's devoted assistance and cooperation made this work possible.

Appreciation goes also to my graduate advisor, Dr. Estry Douglas, who provided academic counseling and encouragement throughout my study at Michigan State University. In addition, I want to thank one of my advisory committee members, Dr. Justin J. McCormick, for his concern and invaluable time.

I would also like to thank Chris Beecher for his assistance during the research period. Finally, I would like to thank all of members in our laboratory for their general support and generous friendship.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AAF	2-acetylaminofluorene
AF	2-aminofluorene
4-ABP	4-aminobiphenyl
B(a)P	benzo[a]pyrene
bp	base pair
BPDE	(±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene
DAB	N-N-dimethyl-4-aminoazobenzene
dA-C8-ABP	N-(deoxyadenosin-8-yl)-4-aminobiphenyl
dG-C8-AAF	N-(deoxyguanosin-8-yl)-2-acetylaminofluorene
dG-C8-AF	N-(deoxyguanosin-8-yl)-2-aminofluorene
dG-C8-1-A6-NP	N-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene
dG-C8-ABP	N-(deoxyguanosin-8-yl)-4-aminobiphenyl
dG-N^2-ABP	N-(deoxyguanosin-8-yl)-4-aminobiphenyl
1,6-DNP	1,6-dinitropyrene
E. coli	Escherichia coli
HPLC	high-pressure liquid chromatography
IPTG	isopropyl-β-D-thiogalactoside
1-NOP	1-nitrosopyrene
1-NP	1-nitropyrene
N-AcO-TFA-ABP	N-acetoxy-N-trifluoroacetyl-4-aminobiphenyl
supF	a gene coding for a tyrosine suppressor tRNA
SV40	simian virus 40
TFA	trifluoracetic acid
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase

INTRODUCTION

It is generally believed that a major cause of human cancer is related to exposure to chemical carcinogens present in the human environment. Chemicals such as aromatic amines are widely used in various industries, particularly the manufacturing of dyestuffs. 4-aminobiphenyl (4-ABP), an aromatic amine, has been reported to be responsible for the induction of bladder cancer among industrial workers employed in dye-manufacturing plants (Melick et al., 1955 ; 1971). Recent research shows that humans are also exposed to 4-ABP by smoking cigarettes (Maclure et al., 1989). The concentration of hemoglobin adducts of 4-ABP in the blood of smokers was found to be significantly higher than that in the blood of non-smokers. Various studies by different groups indicate that 4-ABP is mutagenic in *Salmonella typhimurium* (McCann et al., 1975; 1983; Beland et al., 1983), *Escherichia coli* (Pai et al., 1985), and Chinese hamster ovary cells (Bean et al., 1991).

It has been widely recognized that mutation plays a very important role in the multi-step process of cancer development. However, the understanding of the molecular mechanisms by which chemical carcinogens induce mutations in mammalian cells is considerably less advanced. Earlier studies of this question were done by Yang et al. (1987 ; 1988), Mah et al. (1989;1991), and Boldt et al. (1991) in our laboratory with various compounds. Those compounds are (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10 tetra-hydrobenzo [a] pyrene (BPDE), 1-nitrosopyrene (1-NOP), N-acetoxy-2-acetylaminofluorene (N-AcO-AAF), N-acetoxy-N-trifluoroacetyl-2-aminofluorene (N-AcO-TFA-AF), and 1-nitro-6-nitrosopyrene (1-N-6-NOP), respectively. 4-ABP, as a potent mutagen in bacteria (McCann et al., 1975; Beland et al.,1983; Pai et al.,1985), experimental animals (IARC Monographs,I, 1972; Block et al.. 1978) and humans (Melick et al.,1955; 1971), is of interest to investigate the molecular mechanisms of mutations induced by chemical carcinogens. Therefore, this study was to focus on the behavior of 4-ABP binding with DNA. One of the major differences between AF

and 4-ABP structure is that 4-ABP does not have the bridging $-CH_2-$ group between the two benzene rings. The absence of this $-CH_2-$ group allows for better flexibility of the two benzene rings allowing them to twist along the bond connecting the two rings. This study was aimed at establishing a correlation between mutations and binding compounds by comparing the frequency and the kinds of mutations induced by a series of structurally related chemical carcinogens, as well as their specific locations in the target gene.

In these experiments a shuttle vector, pS189, was used. The reason for choosing pS189 was that it carries a *supF* gene as the target and is capable of replicating in mammalian cells and bacteria (Seidman et al., 1989). One advantage of the shuttle vector assay is that its small size makes it possible to isolate newly mutated genes and to identify them at the sequence level. Another advantage of this assay is that *supF* gene is highly responsive to every kind of mutation, i.e., single base changes at almost any site in the tRNA structure region results in a detectable phenotypic change (Kraemer et al., 1989). The human embryonic kidney cell line, 293, served as the eukaryotic host for the shuttle vector (Graham et al., 1977). *Escherichria coli* SY204 was used as the bacterial host indicator (Sarkar et al., 1984).

In order for 4-ABP to be carcinogenic, it must undergo a conversion to reactive metabolites so that it can react with the macromolecules of the target tissue (Kadlubar et al., 1977).. Therefore, N-acetoxy-N-trifluoroacetyl-4-aminobiphenyl, a reactive form of 4-ABP, was used to treat the plasmid, pS189. It formed a major DNA adduct, N-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8-ABP) and a minor DNA adduct; N-(deoxyadenosin-8-yl)-4-aminobiphenyl (dA-C8-ABP) (Figure 1). These adducts are similar to the adducts obtained *in vivo* (Beland et al.,1983 ;1985). Our results showed that the frequency of *supF* mutant induction increased with the number of 4-ABP adducts per plasmid. By comparing 4-ABP with a series of structurally related compounds used in earlier studies in

Figure 1. *In vitro* interaction of N-acetoxy-N-trifluoroacetyl -4-aminobiphenyl with DNA results in forming a major N-(deoxyguanosin-8-yl)-4-aminobiphenyl DNA adduct, respectively.

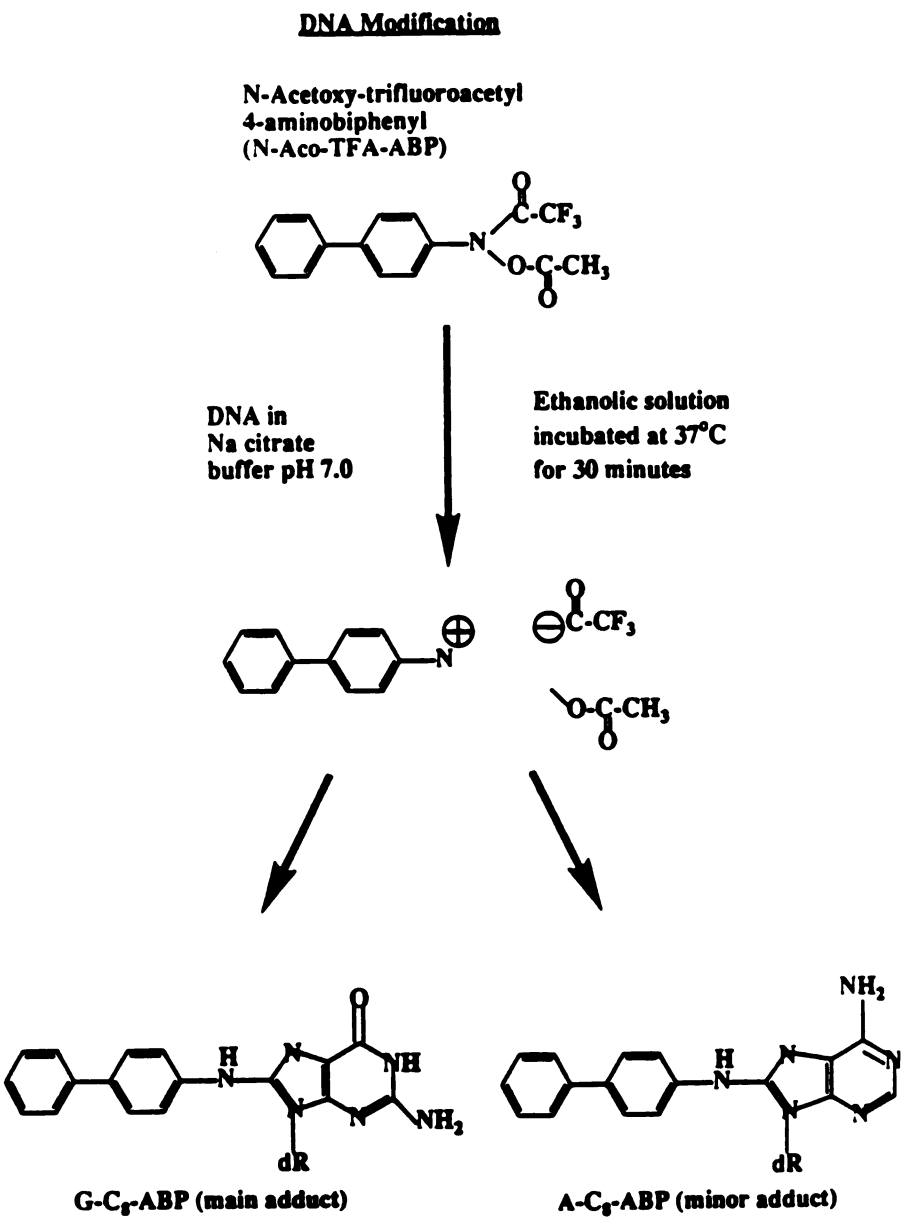

DNA Modification
N-Acetoxy-trifluoroacetyl
4-aminobiphenyl
(N-Aco-TFA-ABP)
DNA in
Na citrate
buffer pH 7.0
Ethanolic solution
incubated at 37°C
for 30 minutes
dR
dR
G-C8-ABP (main adduct)
A-C8-ABP (minor adduct)

Figure1.

our laboratory, the frequency of mutants induced per 4-ABP adduct was found to be ~1.5 times lower than that per 1-N-6-NOP adduct (Boldt et al., 1991) and 3.6 times lower than that per BPDE adduct (Yang et al., 1987). The frequency was almost equal to that per 1-NP adduct (Yang et al., 1988) and per AF adduct (Mah et al., 1989). The mutant frequency per 10^4 plasmids for plasmids carrying 40 carcinogen residues was about 35 for ABP and 50 for AAF, although the mutant frequency per 10^4 plasmids for both 20 ABP residues and 20 AAF residues was the same (~12.5) (Mah et al., 1991). Most of the mutations induced with 4-ABP were base substitutions. Among these base substitutions, 85% involved G·C base pairs which were mainly G·C->T·A transversions. These observations are the same as those seen in earlier studies with AAF, AF, BPDE, 1-NP, and 1-N-6-NOP adducts (Mah et al., 1989;1991; Yang et al., 1987;1988; Boldt et al., 1991) in our laboratory. The other 15% of base substitutions involved A·T base pairs which were mainly A·T->T·A transversions. Earlier studies found only 13% of the base substitutions involved A·T base pairs for 1-NP adducts (Yang et al., 1988) and 10% for BPDE adducts (Yang et al., 1987). Although there are some common "hot spots" in the spectra of induced mutations by various adducts, each compound had its own characteristic mutation spectrum in the *supF* gene (Yang et al., 1987;1988; Mah et al., 1989 ;1991; Boldt et al., 1991).

The objectives of this study were (1) to investigate the specific kinds of mutations induced by ABP adducts at the sequence level when a shuttle vector replicates in human cells; (2) to determine the specific locations of these mutations in the DNA sequence of the target gene; (3) to compare the spectra of mutations induced by ABP adducts in the *supF* gene with those induced by structurally-related carcinogens (N-AcO-AAF, N-AcO-AF, BPDE, 1-NOP, 1-N-6- NOP) in the same assay.

The spectra of mutations induced by structurally-related carcinogens was investigated by my colleagues in our laboratory. A comparison of the spectra of mutations induced by these carcinogens

would be helpful for us to understand more about the mechanisms by which these chemical carcinogens cause mutations. Chapter I gives a brief overview of ABP induced carcinogenesis and mutagenesis and of the assay system used in the present study. Chapter II describes the details on materials and methods. Chapter III presents the experimental results. Chapter IV gives a discussions of the results.

CHAPTER I

LITERATURE REVIEW

A. IMPORTANCE OF AROMATIC AMINES IN CHEMICAL CARCINOGENESIS

Many aromatic amines are important materials in the manufacture of dyes, drugs, plastics and pesticides. After the discovery of aniline in 1826, aromatic amines soon became commercial products. In 1895 Rehn reported that the occurrence of urinary bladder cancer among the dyestuff workers was related to exposure to aniline because aniline was used as an intermediate in the production of magenta. Later Leichenstern (1898) suggested that 2-naphthylamine might be a possible inducer of urinary bladder cancer. Subsequent research proved that aniline was not the causative agent, while other aromatic amines, such as 2-naphthylamine, benzidine and 4-aminobiphenyl were actually responsible for the bladder tumor induction in workers in dyestuff industries (Hueper et al., 1938; Walpole et al., 1954; Bonser et al., 1956; Hueper, 1969). Although the hazardous nature of these compounds has been known since the beginning of the century, their production in some countries continued as late as 1972. Whereas the production of the most hazardous compounds has now been stopped, other aromatic amines are being produced continuously.

Fischer (1906) observed that Scarlet Red caused a hyperplastic reaction in rabbit skin epithelium. Between 1908 and 1911 Scarlet Red and O-aminoazotoluene (2',3-dimethyl-4-aminoazobenzene) were applied in clinical situations to accelerate wound healing (Shear, 1937). Later Schmidt et al. (1924) reported that adenomatous growths were observed in the livers of mice fed Scarlet Red. In the mid-1930's Yoshida (1933) and Sasaki et al. (1935) were successful in inducing liver carcinomas in rats by feeding them with O-aminoazotoluene for a period of time. Later,

Kinosita (1937) reported that N-N-dimethyl-4-aminoazobenzene (DAB), an isomer of O-aminoazotoluene, was a strong hepatocarcinogen in rats. Hueper et al. in 1938 administered 2-naphthylamine into dogs for 20-26 months, 13 out of 16 dogs developed bladder tumors. This confirmed the carcinogenic nature of 2-naphthylamine. In 1940, 2-acetylaminofluorene (AAF), a potentially valuable insecticide, was studied for toxicity in laboratory animals. Wilson et al. (1941) showed that malignant tumors in diverse organs were developed in rats fed with AAF. After then, AAF was eliminated from commercial use. AAF as a model carcinogen was studied for the mechanisms of chemical carcinogenesis.

4-aminobiphenyl, a rubber antioxidant and a dye intermediate, was manufactured on a large scale between 1935 and early 1955. In the mid-1950's, Melick et al. first reported that the risk of human bladder cancer was associated with exposure to 4-aminobiphenyl. This report was based on the fact that 19 of 171 men exposed to 4-aminobiphenyl between 1935 and 1955 developed bladder tumors (Melick et al., 1955). This observation prompted discontinuation of 4-aminobiphenyl production to prevent widespread use of the chemical. Melick et al. further reported in 1971 that 53 out of 315 men exposed to 4-aminobiphenyl had bladder tumors. These studies strongly indicated that occupational exposure to 4-aminobiphenyl was associated with bladder cancer. However, epidemiological data could not indicate a correlation between the tumor frequency and the dose of the carcinogen. Aromatic amines were then extensively studied for their carcinogenicity in animals and all these compounds were found to be carcinogenic in both human and small rodents, except for arsenic derivatives (Tomatis et al., 1978).

4-aminobiphenyl was tested for carcinogenicity by oral administration in rabbits, dogs and mice. Bladder papillomas were observed in rabbits (IARC Monographs I, 1972). Bladder carcinomas were found in dogs (Block et al., 1978), and neoplasms were induced at various sites in mice (Schieferstein et al., 1985). After being

administrated to rats, 4-aminobiphenyl induced tumors of the mammary gland and intestine (IARC Monographs I, 1972). Animal studies for carcinogen testing were usually time consuming and very expensive, therefore a rapid screen method was needed. Since the majority of the chemicals (90%) that cause mutations in bacteria also produce cancer in animals, one such rapid screen test was the Ames test which was used extensively as the first test in screening for human carcinogens (McCann et al.,1975). 4-aminobiphenyl was also found to be mutagenic in bacteria and to induce prophage (IARC, Monographs 6, 1987). Nowadays, 4-aminobiphenyl, as a cigarette component, continues to expose humans. Many investigators found that the concentration of hemoglobin adducts of 4-aminobiphenyl in the blood of smokers was significantly higher than that in non-smokers (Maclure et al., 1989; Ronco et al.,1990). One study showed that the level of 4-aminobiphenyl in adducted hemoglobin was affected by both genetic susceptibility and environmental exposure (Vineis et al.,1990). Bartsch et al. (1990) further indicated that people who smoke black tobacco (air-cured) have higher levels of ABP hemoglobin adducts than those who smoke blond tobacco (flue-cured). Coghlin et al. (1991) reported that 4-aminobiphenyl could cross the human placenta and bind to fetal hemoglobin.

B. REACTIVE INTERMEDIATES OF 4-AMINOBIPHENYL BOUND TO MACROMOLECULES OF THE TARGET TISSUE

Aromatic amines induce tumors which appear at sites distant from the sites of administration (Miller and Miller,1967). This fact indicated that aromatic amines actually are metabolized into an active form in any animal species, and then the active form interacts with crucial cellular components in some manner in order to induce cancer in that species. The initial hypothesis proposed to explain the carcinogenic activity of aromatic amines was called "ortho-hydroxyamine hypothesis" (Clayson, 1962). However, later studies proved that this hypothesis was incorrect because several ortho-hydroxyaromatic amines were discovered to be less

carcinogenic than the parent compounds (Clayson et al., 1976). The evidence that N-hydroxylation was the initial activation step (Miller et al., 1961) was first discovered in Miller's laboratory by studying the carcinogen AAF. Both N- and ring-hydroxylation of 2-acetylaminofluorene (AAF) were found in urine of rats fed by AAF. However, unlike ring-hydroxy-AAF, N-hydroxy-AAF was found to be more carcinogenic than the parent amide in the rat. Therefore ring-hydroxylation was considered to represent an inactivating step in carcinogenesis by aromatic amines. Later, Miller et al. (1964) proved that all species susceptible to AAF carcinogenesis excreted N-hydroxy-AAF in their urine, suggesting that N-hydroxylation was an activation step in carcinogenesis by aromatic amines. N-hydroxylation of AAF was not detected in the urine of guinea pigs, which are resistant to AAF carcinogenesis. However, when N-hydroxy-AAF was administrated to guinea pig by feeding, adenocarcinomas of the small intestine were found; sarcomas were found when given by injection.

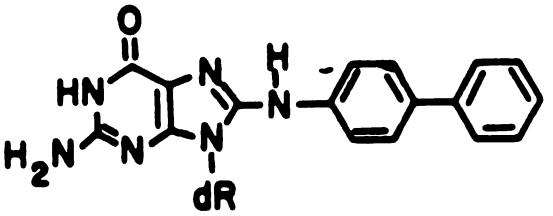
It has been proven that 4-aminobiphenyl has to become N-hydroxylated to an active form, in order to be carcinogenic (Kadlubar et al., 1977). The N-oxidation reaction is catalyzed by tissue enzymes in the hepatic endoplasmic reticulum in the presence of NADPH and O_2. The reactive form of N-hydroxy-4-aminobiphenyl is transported into the circulation and excreted in urine as glucuronides. At pH higher than 7, the glucuronides of N-hydroxy-4-aminobiphenyl are relatively stable. At pH 5 or 6, the hydrolysis of the N-glucuronides greatly increases to yield electrophilic ultimate carcinogens in the urinary bladder lumen. These electrophilic ultimate carcinogens are transported to critical cellular macromolecules by unknown mechanisms. When N-hydroxy-4-aminobiphenyl was reacted with DNA at pH 5, three DNA adducts formed (Figure 2). By comparing these three DNA adducts formed *in vivo* and *in vitro*, N-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8-ABP) was found to be the major adduct which accounted for 80% of the total binding. The second adduct, N-(deoxyadenosine-8-yl)-4-aminobiphenyl, was characterized as making up 15% of the binding.

The third adduct, N-(deoxyguanosin-N2-yl)-4-aminobiphenyl, comprised 5% of the binding (Beland et al., 1983). In contrast to what I found when I reacted N-AcO-TFA-ABP with DNA *in vitro* , dG-N2-ABP accounted for the second most abundant adduct and dA-C8-ABP was identified as the third most abundant adduct *in vivo* . 4-ABP could also bind to hemoglobin to form 4-aminobiphenyl-hemoglobin adducts.

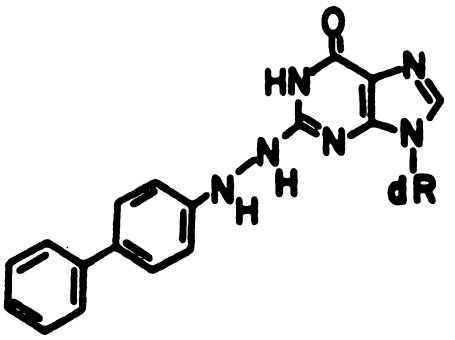
C. 4-AMINOBIPHENYL DNA ADDUCTS AS MUTAGENIC LESIONS

A tumor cell is a persistently dividing cell which cannot be controlled by the host. The transformation of normal cells into tumorigenic cells is a multi-step process. Much evidence indicates that mutations play an important role in cellular transformation and carcinogenesis. However, because numerous kinds of chemical carcinogens exist, the molecular mechanisms for inducing mutations have not been very well understood. There has been great difficulty in fully explaining the mechanisms of cancer formation. One hypothesis called somatic mutation was proposed in 1914 and was widely accepted in the past. It describes that the initiating event in any cancer development is a single chromosomal or mutational event in somatic cells. This hypothesis later became a controversial issue because it lacked experimental support. When Burdette reviewed the hypothesis in 1955, he concluded that the correlation between mutagenicity and carcinogenicity did not exist. A major breakthrough in the understanding of the mechanisms of chemical carcinogenesis came from the discovery of carcinogen metabolic activation step in Millers' laboratory. Their studies indicated that most chemical carcinogens required metabolic activation in order to bind readily to cellular macromolecules. This important insight plus the development of an effective short-term tests for mutagenesis made it possible to study the mutagenic action of diversified chemical carcinogens (Maher et al., 1968;1970; McCann et.al., 1975).

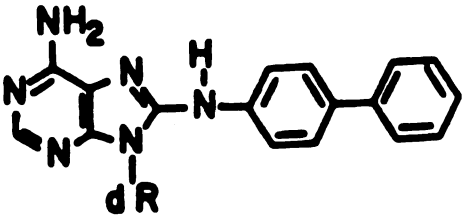
Figure 2. Structures of three aminobiphenyl-DNA adducts.



dG-C8-ABP



dG-N^2-ABP



dA-C8-ABP

Figure 2.

N-hydroxy-4-aminobiphenyl, a reactive form of 4-aminobiphenyl, forms DNA adducts in the bladder epithelium. The major adduct is the guanine C-8 adduct. This DNA adduct causes a DNA conformational change. This change affected DNA replication and repair. Broyde et al. (1985) investigated the conformation of 4-aminobiphenyl that was modified at dCpdG sequence by minimizing semi-empirical potential energy calculations. Two conformers in A or B and Z helices were shown. One conformer was a carcinogen-base stacking which was considered to be a low energy state. Another conformer was base-base stacking. Broyde et al. suggested that if carcinogen was bound to DNA in the A or B form in the helix exterior of the large groove this was considered to be a favored state in random sequence DNA. The favored state might escape from repair because this state causes no distortion. Broyde et al. also pointed out that when DNA was unwound at or near the replication fork, a carcinogen-base stacked state was formed by rotating the C5'-O5' bond. If DNA replication failed to operate faithfully, it could result in a mutagenic outcome. This theoretical calculation proved that changing DNA conformation by 4-aminobiphenyl binding might lead to mutation induction.

When 4-aminobiphenyl was administered to male beagle dogs, the aminobiphenyl-modified DNA adducts were found to be predominantly C8 of deoxyguanosine adducts. The level of these adducts remained constant for 7 days in the bladder epithelium . It was also found that the total DNA binding correlated with the ability to induce bladder tumors in dogs (Beland et al., 1983). 4-aminobiphenyl was also shown to be mutagenic in *Salmonella typhimurium* (McCann et al., 1975). When *Escherichia coli* virus M13mp10 that carried aminobiphenyl lesions was studied, this kind of lesion caused mainly base pair substitution mutations in an SOS-processing environment (Lasko et al.,1988). Aminobiphenyl lesions also induced mutation in human fibroblasts, DNA strand breaks, and unscheduled DNA synthesis in cultured rodent cells (IARC Monographs 7 1988).

D. THE VALUE OF USING THE PS189 PLASMID ASSAY IN H293 CELL LINE

The molecular mechanisms for the induction of mutations in mammalian cells are not well understood. Attempts to explain the mechanisms involved have been hampered in the past because of an inability to isolate and to analyze newly mutated genes at the sequence level. However, the development of shuttle vectors, plasmids, that can replicate in mammalian cells and in bacteria, solved this problem (Sarkar et al., 1984). Several shuttle vectors allowed replication of bacterial plasmid DNA in mammalian cells. One of these shuttle vectors, called lac I shuttle vector, was extensively characterized and widely used to investigate spontaneous and induced mutations in both bacteria and mammalian cells (Calos et al., 1983; Lebkowski et al., 1984; Razzaque et al.,1984). In our experiment a 5337 bp recombinant plasmid called pS189 was used (Seidman, 1989). It was a deletion derivative of pZ189 which carried a bacterial tyrosine amber suppressor tRNA gene (*supF*) (Seidman et.al., 1985). The small size (167 bp) of the gene permited the determination of the entire DNA sequence. The *supF* gene is also highly responsive to every kind of mutation. Any change in 64 of the 85 nucleotides that make up the tRNA structure have been shown to result in a detectable phenotypic change (Kraemer and Seidman, 1989). This gene, which serves as a mutagenesis marker, is flanked by the gene for ampicillin resistance and the bacterial origin of replication facilitating its replication in bacteria. The plasmid carrying target DNA sequences can be exposed to mutagens or carcinogens either *in vitro* before transfection, or *in vivo* after introducing the DNA into the mammalian cells. The progeny plasmids can then be rescued. Mutants in bacteria are detected and analyzed at the sequence level. Deletions, insertions, and gross alterations which altered gel mobility of the plasmid are easily detected and those plasmids that do not show deletions, etc., of the *supF* gene can be sequenced.

CHAPTER II

MATERIALS AND METHODS

A. MATERIALS

1. PLASMID

The 5337bp shuttle vector, pS189, is a deletion derivative of pZ189 (Seidman, 1989). It contains the tyrosine amber suppressor tRNA gene (*supF*) flanked by two genes essential for recovery in *E. coli* . These two genes are the ampicillin gene (β-lactamase) and the bacterial origin of replication. PS189 also contains the origin of replication and large T-antigen gene from Simian virus-40 (SV-40). These facilitate pS189 replication in mammalian cells (Figure 3). The tyrosine amber suppressor tRNA gene (*supF*) contains a G to C transversion mutation in the anticodon of $tRNA_I^{tyr}$. The anticodon in *E. coli* tyrosine $tRNA_I^{tyr}$ is GUA. This G to C mutation changes GUA to CUA which allows incorporation of tyrosine at the amber chain termination (nonsense) codon, UAG. *SupF* thus functions as a suppressor tRNA, enabling readthrough of the UAG stop codon. The *supF* gene consists of a portion of the promoter region (base pairs 24-58), a precursor RNA region involved in RNA processing (base pairs 59-98), the suppressor tRNA sequence (base pairs 99-183), and a 3'-flanking region (base pairs 184-200), respectively. There are several advantages in using *supF*. The first is its size. The relatively small size makes it posible to study mutation of the target gene at the sequence level. The second advantage is that the *supF* gene is highly responsive to all kinds of mutations, i.e, base substitutions, frameshifts, deletions, insertions, rearrangements, etc. A change in any one of at least 64 nucleotides out of the total 85 nucleotides making up the tRNA structure has been shown to result in a detectable phenotypic change (Kraemer and Seidman, 1989). The third advantage is that plasmids

Figure 3. pS189 shuttle vector.

The 5337-base-pair vector contains the ampicillin resistance gene (β-lactamase), the pBR327 origin of replication (ori), and the *E. coli supF* tyrosine suppressor tRNA gene from pAN7; and the origin of replication and T-antigen coding region from SV40 (clock-wise from SV40 nucleotides 2533 to 346). Arrows indicate the direction of transcription.

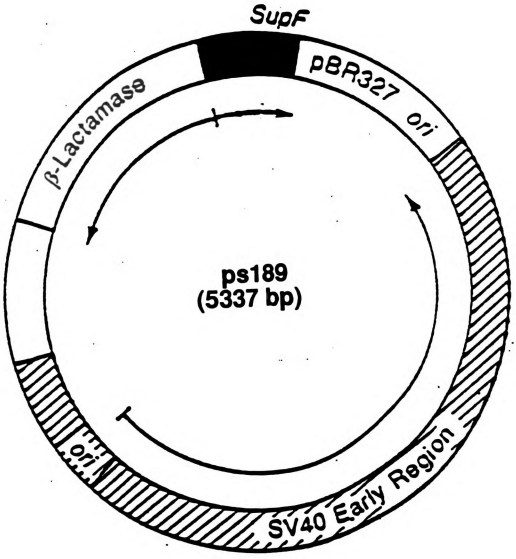

SupF
pBR327 ori
β-Lactamase
ps189
(5337 bp)
ori
SV40 Early Region

Figure 3.

containing a mutant *supF* gene can be identified and isolated by transforming them into bacterial cells. The fourth advantage is that deletions, insertions, and gross rearrangements in *supF* gene are easily detected by gel mobility change.

2. CELLS

1) H293 Cell:

A human embryonic kidney cell line, 293, transformed by adenovirus 5 DNA fragments served as the eukaryotic host. It was obtained from Dr. Michele Calos (Stanford University, Stanford, CA). The cells were grown in Eagle's minimal essential medium supplemented with 0.2mM L-serine, 0.2mM L-aspartic acid, 1mM sodium pyruvate, 10% fetal calf serum (GIBCO) and antibiotic (Yang, et al., 1987).

2) *E. coli* SY204:

The ampicillin-sensitive indicator bacterial host was *E. coli* SY204 carrying an amber mutation in the β-galactosidase gene and in the tryptophan gene (Sarkar et al.,1984). The *E. coli* SY204 was obtained from Willian C. Summers (Yale University, New Haven, CT).

3. REAGENTS

1) LB (Luria-Bertani) Medium:

10 g Bacto-Tryptone, 5g Bacto-Yeast Extract, and 10 g NaCl were mixed and then disolved in 800 ml ddH_2O. The solution was adjusted to pH 7.5 with NaOH and the volume was adjusted to 1 liter with ddH_2O. The solution was then autoclaved and stored at 4^oC.

2) STE Solution:

STE solution contained 0.1 M NaCl, 10 mM Tris-Cl, and 1 mM EDTA pH 8.0. The Solution was made from a 2 M NaCl stock solution, a 1 M Tris-Cl, pH 8.0, stock solution, and a 0.5 M EDTA, pH 8.0, stock solution.

3) Ampicillin Stock Solution:

Sodium salt of ampicillin was disolved in ddH_2O at 25 mg/ml. The solution was sterilized by filtration and stored in aliquots at -20^oC.

4) Lysis solution I:

Lysis solution I contained 50 mM Glucose, 25 mM Tris-Cl, and 10 mM EDTA, pH 8.0. The following stock solutions were used: 1M Glucose, 1M Tris-Cl, pH 8.0, and 0.5 M EDTA, pH 8.0.

5) Lysis solution II:

Lysis solution II contained 0.2 N NaOH and 0.1% SDS. This solution was made from a 10 N NaOH stock solution and a 20% SDS stock solution. The solution was made fresh before it was to be used.

6) Potassium Acetate solution II (for lysing):

11.5 ml Glacial Acetic Acid and 60 ml 5 M Potassium Acetate were added to 28.5 ml H_2O to make a 100 ml solution of 3 M Potassium and 5 M Acetate.

7) Solution III:

Solution III contained 50 mM Tris-Cl, 0.01 M EDTA, pH 8.0, and 0.01 M NaCl. The solution was made from a 1 M Tris-Cl, pH 8.0, stock solution, a 0.5 M EDTA, pH8.0, stock solution, and 2 M NaCl stock solution.

8) TE (pH 8.0):

TE solution contained 10 mM Tris-Cl and 1 mM EDTA, pH 8.0. The solution was made from a 1 M Tris-Cl, pH 8.0, stock solution and a 0.5 M EDTA, pH 8.0, stock solution.

9) Ethidium Bromide stock concentration:

10 mg Ethidium Bromide was weighed and disolved in 1 ml ddH_2O to make a final 10 mg/ml concentration.

10) SOB Medium:

20 g Bacto Tryptone, 5 g Bacto Yeast Extract, 0.6 g NaCl, and 0.2 g KCl were weighed separately and mixed together. 800 ml ddH_2O was added to the mixture and the mixture was stirred until completely dissolved. The solution was adjusted to pH 6.8-7.0 and ddH_2O was added to make a final volume of 1 liter. The solution was then autoclaved and 10 ml 2 M Mg2+ stock solution was added. Mg2+ solution was made from 1 M $MgCl_2 \cdot 6H2O$ and 1 M $MgSO_4 \cdot 7H_2O$ stock solutions. The final solution was sterilized by filtration and stored at 4°C.

11) TFB (500 ml):

5 ml 1 M KMES* pH 6.2, was measured into a 1000-ml beaker. 6.045 g RbCl, 4.453 g $MnCl_2 \cdot 4H_2O$, 0.735 g $CaCl_2 \cdot 2H_2O$, and 0.401 g $HACoCl_3$ were added. The solution was mixed, adjusted to 500 ml, and then stored at 4°C.

KMES* (2-N-Morpholinoethanesulfonic Acid):

1 M stock solution was adjusted to pH 6.2-6.3 with 1 M KOH and then sterilized by filtration and stored at -20°C.

12) DTT (for transfection only):

1 M dithiothreitol was made using 40 mM potassium acetate (pH 6.0). The solution was aliquoted into 0.5 ml Eppendorf tubes and stored at -20°C. Each aliquot was used once and discarded.

13) DMSO:

The degassed DMSO was aliquoted into 0.5 ml Eppendorf tubes and stored at -20°C. Each aliquot was used once and discarded.

14) X-Gal agar plates:

7 g Bacto agar was weighed and added into 500-ml of LB medium. The solution was then autoclaved and cooled to 50°C in a 50°C water bath. 1 ml X-Gal with stock concention 20 mg/ml, 0.5 ml IPTG with stock concention 20 mg/ml, and 1 ml Ampicillin with stock concention 25 mg/ml were added before the solution was poured into plates. The plates were made 0.5-1 cm thick. These plates could be stored at 4°C for up one month.

20 mg/ml X-Gal:

220 mg X-Gal was dissolved in 11 ml dimethylformamide and sterilized by filtering.

20 mg/ml IPTG:

120 mg IPTG was dissolved in 6 ml distilled H_2O and sterilized by filtering.

25 mg/ml Ampicillin:

2.5 g of Ampicillin was dissolved in 100 ml ddH_2O, filter-sterilized, and aliquoted into 10 ml vials.

15) 2x Hepes Buffer Solution:

10 g HEPES, 16 g NaCl, 0.74 g KCl, 0.22 g Na_2HPO_4, and 2 g Dextrose were dissolved into 800 ml ddH_2O. The solution was adjusted to pH 7.05 with NaOH, and made to 1 liter with ddH_2O. The solution was filter-sterilized , aliquoted ,and stored at -20°C.

16) 2 M $CaCl_2$:

22.2 g $CaCl_2$ powder was dissolved in ddH_2O, and the volume was brought to 100 ml. The solution was filter-sterilized, aliquoted, and stored at -20°C.

17) Complete Medium:

10% Fetal calf serum and 1:300 Pen./Strep. solution was added to Eagle's MEM medium.

18) 25X Strength Vogel/Bonner Minimal Media (VBMM Plates):

5.0 g $MgSO_4 \cdot 7H_2O$, 50.0 g citric acid $\cdot H_2O$, 250.0 g K_2HPO_4, and 87.5 g $NaNH_4HPO_4 \cdot 4H_2O$ were dissolved into 670 ml dd H_2O. The solution was adjusted to 1 liter with ddH_2O, aliquoted into 500ml bottles, and autoclaved. The solution was diluted to 1X before use.

19) 1X Strength Vogel/Bonner Minimal Media (VBMM Plates):

20 ml 25X VBMM solution was mixed with 480 ml dd H_2O to make a 1X VBMM solution. 10 ml of a 25% glucose solution and 0.5 g casamino acids were added to this solution to make a final concentration of 0.5% glucose and 0.05 g casamino acids. The solution was autoclaved for 10 mimutes at 121°C and removed immediately.

B. METHODS

1. PS189 PLASMID PREPARATION

Plasmid was prepared by using an alkaline lysis procedure (Maniatis et al., 1982) and purified by ethidium bromide-CsCl density gradient centrifugation. 4-ABP adducts were introduced into the plasmid and characterized by the methods described detail by Tamura and King (1989).

1) Growth of Bacteria and Amplification of the Plasmid:

10 ml of LB medium containing the appropriate antibiotic (e.g. ampicillin used at 50 μg/ml) was inoculated with a single bacterial colony and then incubated overnight at 37°C with vigorous shaking. The following morning 1 ml of overnight culture was inoculated into 25 ml of fresh LB medium in a 100-ml flask containing the appropriate antibiotic and the mixture was incubated at 37°C with vigorous shaking until the culture reached the late log phase (about 2-3 hours, OD=0.6). 25 ml of the late log culture was then inoculated into 500 ml of LB medium, prewarmed to 37°C and containing the appropriate antibiotic, in a 2-liter flask and incubated at 37°C with vigorous shaking for exactly 2.5 hours. The OD_{600} of the culture would be approximately 0.4. Chloramphenicol, 85 mg, was added to the 500 ml culture to reach a final concentration of 170 μg/ml and then the solution was incubated at 37°C with further vigorous shaking for 12-16 hours. The bacterial cells were harvested by centrifugation at 2000 rpm for 15 minutes at 4°C. The supernatant was pipeted into a waste container. The bacterial cells were washed in 100 ml ice-cold STE solution and separated by centrifugation at 2000 rpm for 15 minutes at 4°C. The bacterial cell pellets were resuspended in 8 ml of lysis solution I containing 6 mg/ml lysozyme. This suspension was transfered to Beckman SW27 polyallomer tube and then incubated at room temperature for 5 minutes. 16 ml fresh lysis solution II was added

and mixed with the suspension. The solution was left in a ice bath for 10 minutes. 12 ml of a ice-cold 5 M potassium acetate solution (pH 4.8) was added to the solution. The solution was mixed and then put on ice for 10 minutes. Centrifugation at 15000 rpm for 25 minutes at 4^{o}C caused the DNA, cell and bacterial debris to form a tight pellet on the bottom of the tube. The supernatant was divided equally into two Corex tubes. 0.6 volumes of isopropanol was added to each tube. The solution was mixed well and left for 15 minutes at room temperature. The plasmid DNA was collected by centrifugation in a Sorvall rotor at 15000 rpm for 30 minutes at room temperature. The supernatant was pipeted off. The DNA pellets were dissolved in a total of 6 ml of TE buffer (pH 8.0). The plasmid DNA was then purified by centrifugation to equilibrium in cesium chloride-ethidium bromide density gradients.

2) Purification of Plasmid DNA by Centrifugation to Equilibrium in Cesium Chlorided-Ethidium Bromide Density Gradients

The volume of plasmid DNA solution was measured. Exactly 1.06 g of solid cesium chloride was added for each milliliter of DNA solution. The solution was mixed gently until all of the salt was dissolved. 0.8 ml of ethidium bromide solution (10 mg/ml) was added for each 10 ml of cesium chloride solution. The cesium chloride solution with the protein aggregates was transfered to a tube suitable for centrifugation in a Beckman Type-50 or Type-65 rotor. The contents were centrifuged at 40000 rpm for 2 days at 20^{o}C. Two bands of DNA could be observed in UV light. The upper band consisted of linear bacterial DNA and nicked circular plasmid DNA and the lower band consisted of closed circular plasmid DNA. A Pasteur pipet was used to carefully remove the upper band . The cellular DNA was discared. Another Pasteur pipet was used to collect the lower band. The ethidium bromide was removed by adding an equal volume of 1-butanol to the lower band solution. This solution was vortexed and spun in an Eppendorf centrifuge for 1 minute at room temperature. The lower aqueous phase was transferred to a clean glass tube. This extraction was repeated for

4-6 times until all pink color disappeared from the aqueous solution. The plasmid DNA was dialyzed against water for 2 days. The DNA concentration was determined from the absorption profile, and plasmids were stored at 200-500 μg/ml in TE (pH 8.0) at -20°C.

3) Formation of 4-ABP Adducts on the Plasmid:

Plasmid was prepared and purified as described. Formation of 4-ABP adducts on the plasmid was performed by Dr. N Tamura of the Michigan Cancer Foundation Detroit using the procedures described in detail by Tamura and King. (1990). Briefly, 70 mg DNA was first dissolved in 2-10 mM sodium citrate (pH 7.0). The solution was then added to an ethanolic solution of tritiated N-AcO-TFA-ABP (60 mCi/mmole). The mixed solution was incubated for 30 minutes at 37°C . The unbound compound was removed by four extractions with phenol and two extractions with ether. The modified DNA was isolated by precipitation with ethanol and then dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.0). The number of residues bound per mole of plasmid was calculated from A_{260} absorption profile of the DNA and the specific activity of the carcinogen. The prepared sample was then stored at -20°C until use.

4) Characterization of Kinds of Adducts Formed:

The adducts were characterized by treating with trifluoracetic acid (TFA) as described by Tamura and King (1990). The modified plasmids were incubated with trifluoracetic acid (TFA) for one hour at 70°C. The hydrolysate was dried under vacuum and then dissolved in methanol. The products of hydrolysis were analyzed by reverse phase HPLC on a C18 μBondapak column with a linear elution. A methanol/water (0-100%) gradient was used at a flow rate of 1.0 ml/minute. The tritium content was estimated by liquid scintillation spectrometry and collected at 30 second intervals (Figure 4).

Figure 4. Chromatography profile of adducts derived from ABP-modified pS189 plasmid following hydrolysis with TFA. The plasmid was reacted with [^{3}H]N-AcO-TFA-ABP, as described and analyed immediately by HPLC. The methanol concentration of the solvent is shown by a broken line.

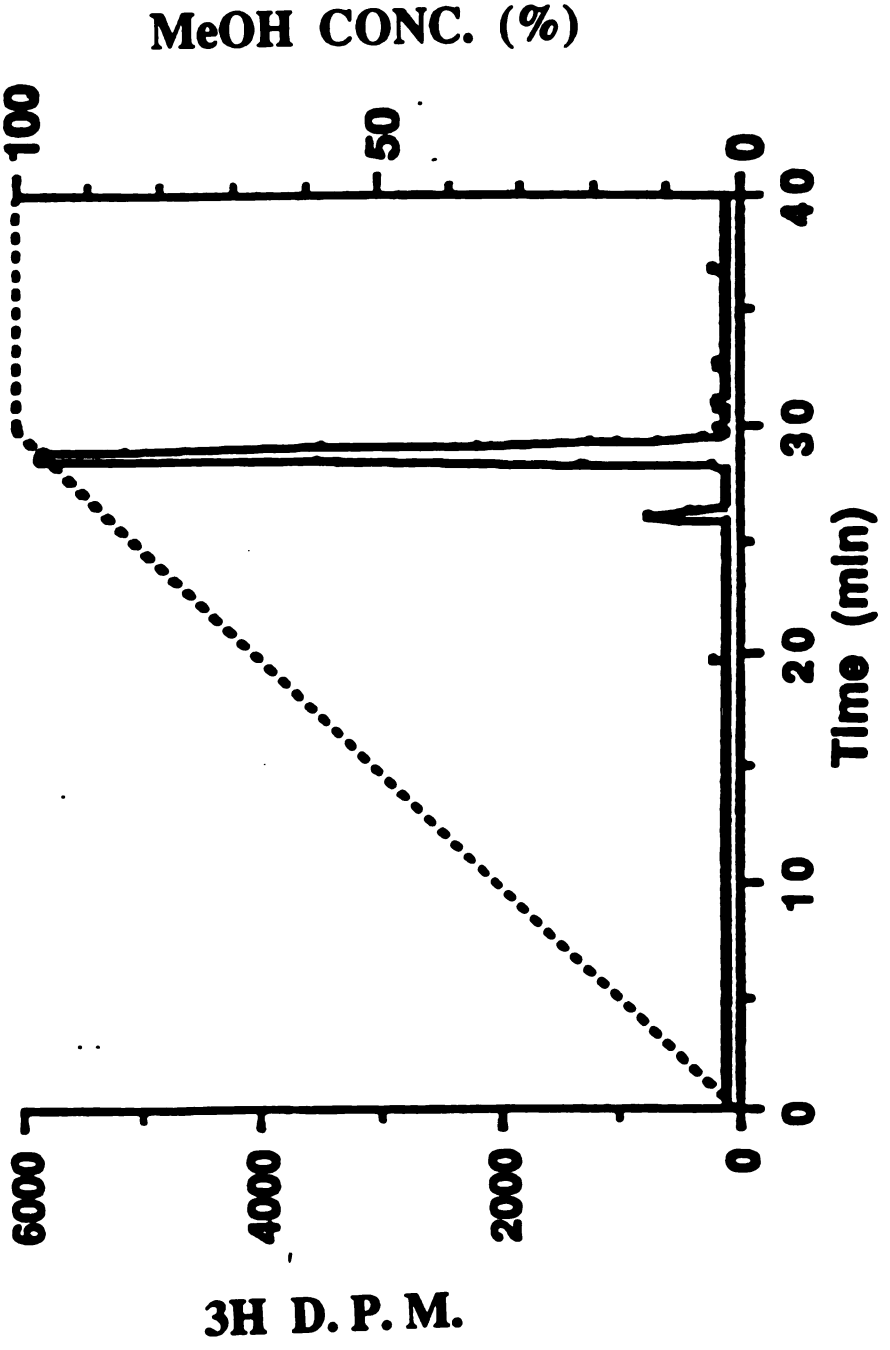


Figure 4.

2. TRANSFECTION OF H293 CELLS AND ISOLATION AND PURIFICATION OF THE PROGENY PLASMIDS

The complete procedures for introducing the treated plasmids into H293 cells by $CaPO_4$ coprecipitation and for rescuing progeny plasmids were described by Yang et al. (1987) (Figure 5A and Figure 5B). Treated plasmids were tested for the relative transformation efficiency before being transfected into human cells.

1) Transfection of H293 Cells by $CaPO_4$ Coprecipitation Methods:

H293 cells were always maintained in exponential growth. The cells were plated onto a series of P_{100} dishes at a density of 2-3x10^6 cells/dish about 24 hours before transfection and then incubated under growth conditions for 24 hours. Transfection cocktails were prepared one hour before transfection. Each dish required 1.5 ml of transfection cocktail. The 1.5 ml of transfection cocktail per dish was adjusted accordingly. First, 82 ml of 2 M $CaCl_2$ was combined with water and the amount of water was determined by the amount of plasmid DNA. Second, 5-10 µg DNA was carefully added, drop by drop, into the above solution. Finally, 650 µl of 2X HeBS was added to the solution. The cocktail was blown through a Pasteur pipet inserted below the surface of the liquid. The cocktail was made and left for 1 hour at room temperature to allow a precipitate to form. The P_{100} dishes were taken out of the incubator and the medium was removed. 1.5 ml of transfection cocktail was added to each P_{100} dish. The cells were exposed to transfection cocktail for 20 minutes at room temperature. The dishes were shaken gently once every 5 minutes. Then, 12 ml of Eagle's MEM medium containing 10% fetal calf serum was added to each dish and the dishes were incubated at 37oC, 5% CO2 for 5-6 hours. The dishes were gently shaken back and forth 20 times once every hour in the incubator. The plates were then taken out of the incubator and the medium was removed. The cells were washed twice using 8 ml of Eagle's MEM medium containing 10%

Figure 5A. The first part of the human cell transfection experiment

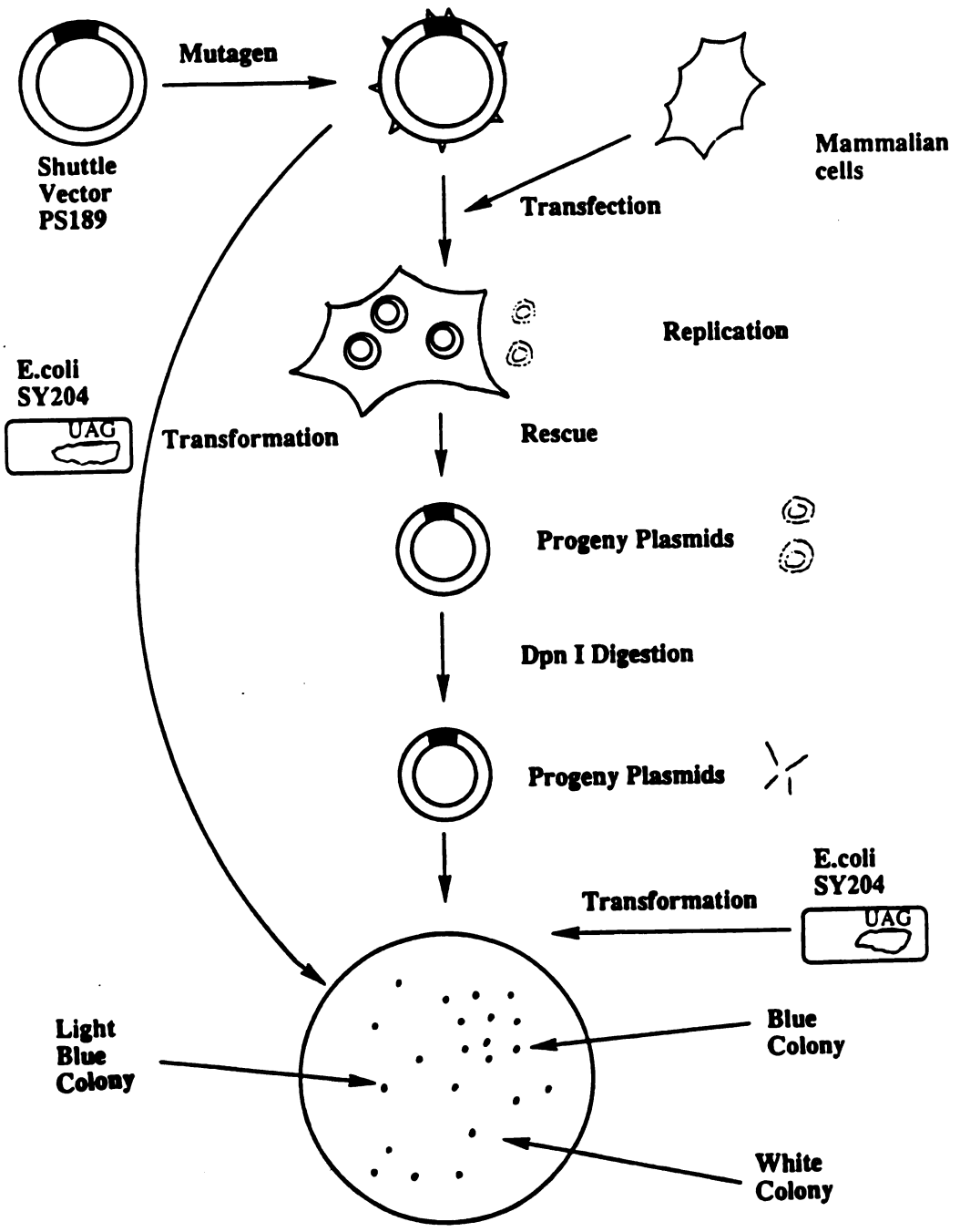
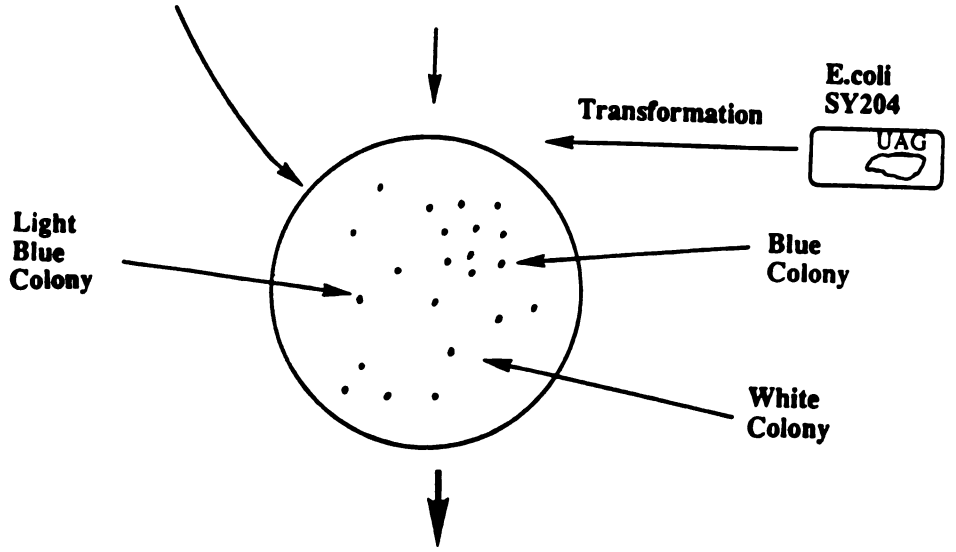

Mutagen
Shuttle
Vector
PS189
Mammalian
cells
Transfection
Replication
E.coli
SY204
UAG
Transformation
Rescue
Progeny Plasmids
Dpn I Digestion
Progeny Plasmids
E.coli
SY204
UAG
Transformation
Light
Blue
Colony
Blue
Colony
White
Colony

Figure 5A.

Figure 5B. The second part of the human cell transfection experiment



Restreak white and light blue colonies on X-gal plate to confirm their phenotype

Restreak mutant colonies on tryptophan minus plate to eliminate bacterial itself mutations

Store mutants and isolate mutant plasmids from white and light blue transformants by Seidman's method

Characterize mutant plasmids by gel electrophoresis

Sequence putative point mutations

Analyze the specific location of DNA changes

Figure 5B.

fetal calf serum and a 300-fold dilution of Pen./Strep. solution and carefully fed with 20 ml of the same medium. The plates were put back in the incubator and cultured at 37°C, 5% CO_2 for 2 days.

2) Recovery of Plasmids by Hirt Extraction:

2 ml of lysing mixture containing 0.6% SDS and 0.01M EDTA was added to each dish and left at room temperature for 20 minutes. In order to distinguish independent mutants with identical mutations from putative siblings derived from a single event, the lysed cells were carefully scraped from each dish separately using a rubber policeman and collected into separate plastic centrifuge tubes. 5 M NaCl was added to the lysed cells to make a final concentation of 1M and mixed well by gently inverting the tube 10 times. The mixture was stored for at least 8 hours at 4°C and spun at 15000 rpm for 30 minutes at 4°C. The plasmid DNA would remain in the supernatant fraction with RNA. The cellular DNA along with SDS and protein would be pelleted at the bottom of the tube.

3) Hirt Supernatant Purification:

An equal volume of phenol was added to the supernatant and the solution was spun at 2000 rpm for 10 minutes at room temperature. The upper layer containing the DNA was collected and treated with RNase (50 mg/ml) (RNase stock:10 mg/ml) for 30 minutes at 37°C. Proteinase K (20 mg/ml) was then added to the mixture to a final concentration of 100 µg/ml. The solution was mixed well and placed in a 55°C water bath for 2-4 hours. The DNA contents were extracted with an equal volume of phenol/chloroform:isoamyl alcohol (1:1 v/v) and spun at 2000 rpm for 10 minutes at room temperature. The upper layer was saved and extracted with an equal volume of chloroform : isoamyl alcohol (24:1). The mixture was spun at 2000 rpm for 10 minutes at room temperature. The top layer, which contained DNA, was saved and mixed with 0.5 volumes of 7.5 M NH_4Ac solution, then EtOH at a volume three times that of the above solution was added and mixed

well. The solution was kept at -20°C overnight or -70°C for a hour. The DNA was then pelletted by centrifugation at 15000 rpm for 25 minutes at room temperature. The DNA pellet was resuspended in 100 ml TE buffer (pH 8.0) and further purified by drop dialysis against TE buffer (pH 8.0) for 2 hours. The purified DNA was treated with Dpn I (2 units Dpn I/mg input DNA) at 37°C for 2 hours to digest any DNA that still had the methylation pattern that was generated during preparation of the original input plasmid in bacteria (Figure 6). After Dpn I digestion the solution was heated at 70°C for 15 minutes to inactivate Dpn I. 1 out of 40 volumes of Dpn I treated DNA solution was treated with Mbo I which digests plasmids that are completely unmethylated after being replicated in the human cells. The plasmids that were treated with Mbo I were tested for ability to transform *E. coli*. The rest of the DNA solution was kept for mutation assay. One twentieth of the total plasmid DNA was used per transformation analysis.

3. TRANSFORMATION OF *E. coli* SY204

Progeny plasmids from H293 cells were transformed into SY204 bacterial cells to analyze the mutant *supF* gene. Bacterial cells containing plasmids with a mutant *supF* gene were selected on X-Gal plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal), isopropyl-β-D-thiogalactoside (IPTG), and ampicillin. The cells with mutant plasmid formed light blue or white colonies. The technique used for bacterial transformation was the same as the one reported by Yang et al. (1987). The three major steps for transformation were making recipient SY204 cells competent, introducing plasmids into the competent cells, and identifying mutants.

1) Making Recipient SY204 Cells Competent:

One single SY204 colony was inoculated into 5 ml SOB medium and cultured overnight with shaking at 225 rpm at 37°C. The overnight culture of SY204 cells was diluted 50 fold in SOB medium

Figure 6. Restriction endonuclease (Dpn I) digestion experiment

Dpn I digestion of plasmid DNA with the bacterial methylation pattern. Dpn I digests DNA with methylated adenine on the 5' GATC 3' sequences. The thicker circles represent plasmids that have replicated in mammalian cells. The thiner cirles are the input plasmids that were prepared from bacteria.

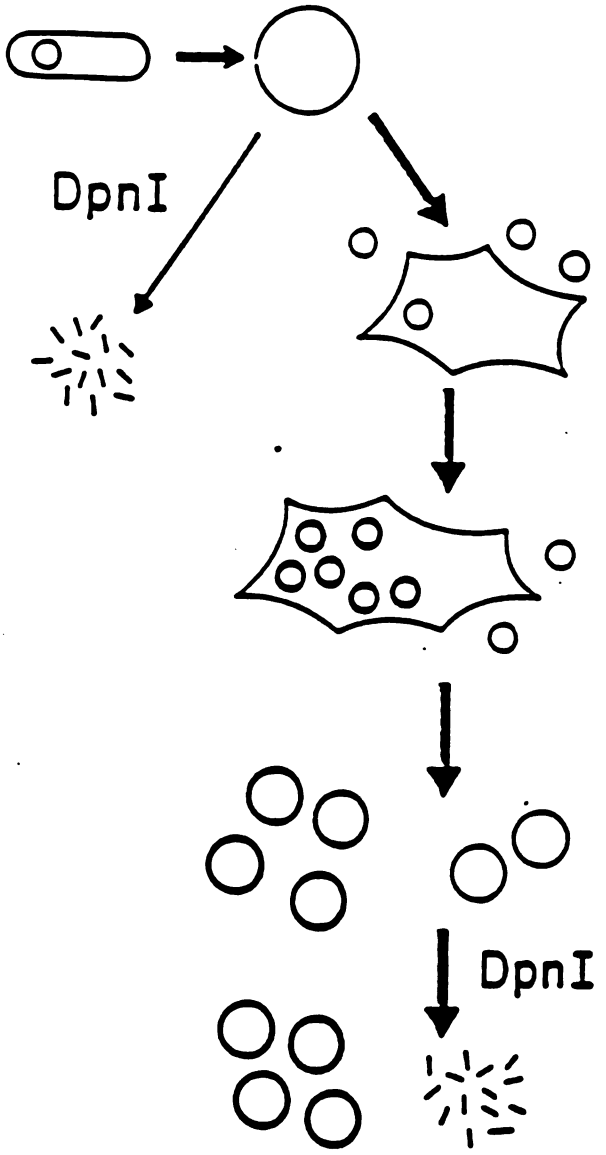


Figure 6.

and cultured again at 37°C for 1.25 hours with shaking at 225 rpm. The OD_{600} of the culture would be approximately 0.25 (~10^8 cells/ml). SY204 cells were collected in 50 ml polypropylene tubes (Falcon 2070) and left on ice for 10-15 minutes. The cells were spun at 2000 rpm at 4°C for 15 minutes. The cell pellets were resuspended in 1/3 the volume of TFB solution and left on ice for 15 minutes. The cells were spun again at 2000 rpm for 15 minutes at 4°C and resuspended in 1/12.5th of the original volume in TFB solution. X·7 ml of fresh stock DMSO was added and the solution was left on ice for 5 minutes (X is the number of individual transformations) to be carried out at one time. The fresh stock DMSO was prepared by aliquoting degassed DMSO into 0.5 ml Eppendorf tubes and storing at -20°C. X·15 ml of stock DTT (1 M dithiothreitol in 40 mM potassium acetate, pH 6.0) was then added and the solution was left on ice for another 10 minutes. Later, X·7 ml of DMSO was added again and the solution was kept on ice for 5 minutes. At this stage the competent cells were ready for transformation.

2) Transformating the Plasmids to Competent Cells:

210 ml of the competent cells were pipeted into chilled polypropylene tubes. 10 ng of the plasmid DNA was added to the competent cells and mixed well. The contents were left on ice for 30 minutes and then heated at 37°C for 2 minutes. The contents were put on ice again for 1-2 minutes. 0.8 ml SOB solution was added and the mixture was incubated for 30-60 minutes at 37°C with shaking at 225 rpm. 200 µl of each dilution was added to the center of an X-Gal plate. The aliquot was spread over the entire surface of the medium by gently moving a sterile, bent glass rod back and forth, while rotating the plate on a wheel. The glass spreader was sterilized by being dipped into a beaker containing 70% EtOH and burning the EtOH in the flame of a bunson burner. The X-Gal plates were incubated overnight at 37°C and colonies were counted. Plasmids containing a mutant *supF* gene could be identified because *E. coli* SY204 containing non mutated pS189 formed blue colonies,

whereas *E. coli* SY204 cells containing mutated pS189 formed white or light blue colonies (Figure 7).

3) Identifying Mutant:

White and light blue colonies were restreaked on X-Gal plates and on plates lacking tryptophan (VBMM plates) to confirm their phenotype (Maher, et al., 1968). White and light blue colonies which did not grow on tryptophan minus plates were considered to be real pS189 mutants, those that did grow were considered to be SY204 mutations. The *supF* mutation frequency was determined by the number of white and light blue colonies divided by total transformants. The relative transformation efficiency of various number of adducts per plasmid was calculated.

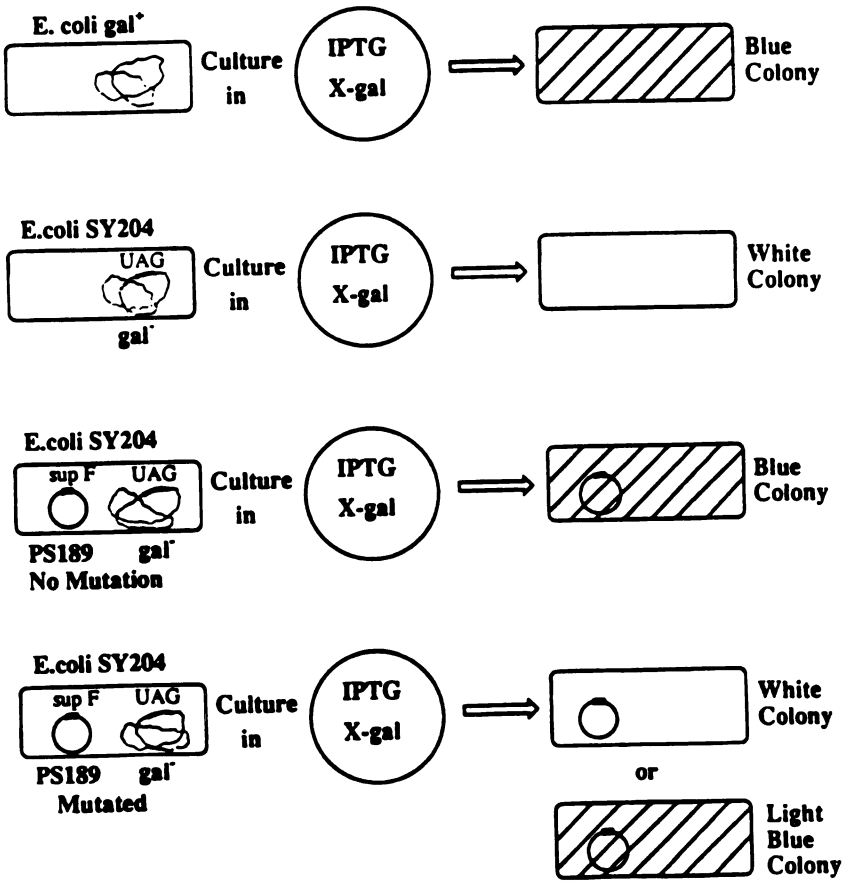
4. DNA PREPARATION FROM MUTANTS AND ANALYSIS ON AGAROSE AND POLYACRYLAMIDE GELS

Mutant plasmids were amplified and purified using a small scale alkaline lysis procedure (Seidman's method) as described by Zagursky et al. (1985). The plasmids were then analyzed by agarose gel electrophesis for altered DNA mobility.

1) Mutants Storage and Small-Scale Isolation of Plasmid DNA

7 ml of LB medium containing 100 µg/ml ampicillin and 0.01 mg/ml Mg^{++} was inoculated with a single colony and incubated at 37°C overnight with vigorous agitation. 1.5 ml of the mutant plasmid culture was pipeted into a 2 ml polypropylene vials containing 0.5 ml sterile glycerol for storage. The solution was mixed well and stored at -80°C for 6 hours before transfering to -135°C. The mutant plasmids could be kept for many years. 5 ml of the culture was pipeted into a 15 ml plastic tube (Falcon#2059) and spun at 2000 rpm for 15 minutes at room temperature. The supernatant was poured off. The cells were resuspended in 500 µl of lysis solution I. The suspension was transfered to a 1.5 ml

Figure 7. Mutated and non-mutated pS189 plasmid identification experiment.



IPTG: β-D-thiogalactoside
(an inducer of β-galactosidase)

X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactoside
(substrate)

Figure 7.

Eppendorf tube and spun for 5 minutes at room temperature. 420 µl of supernatant was pipeted off. The pellet was resuspended and mixed with 200 µl of lysis solution II by inverting 10 times. 150 µl of 5M potassium acetate (pH 5.7-5.8) solution II was added to the solution and mixed by inverting 10 times. The mixture was then left on ice for 5 minutes. The solution was spun for 15 minutes at 4°C. The supernatant was saved and mixed with 50 µl of 2M Tris base. An equal volume of phenol was added to the supernatant, mixed well and then spun for 5 minutes at room temperature. The supernatant was saved in a clean tube. 0.5 ml isopropanol was mixed with the supernatant and left for 20 minutes at room temperature. The mixture was spun for 15 minutes at 4°C and the supernatnat was pipeted off. The pellet was rinsed twice with 200 µl 70%EtOH and dried for 15 minutes under vaccum. The pellet was resuspended in 50 µl of solution III and mixed with 1 µl RNAase A (10 mg/ml). The solution was incubated in a 37°C water bath for 45 minutes. 11.5 µl of 5 M LiAc,1.6 µl of 20% SDS, and 1 µl of 20 mg/ml Proteinase K mixture were added and incubated at 37°C water bath overnight or at 55°C for 2 hours. Phenol extraction was performed one to two times. One phenol/choloform extraction was performed. Then 14 µl of 7.5 M NH_4Ac and 200 µl 100% EtOH were added. The DNA was precipitated for 30 minutes at room temperature and spun for 15 minutes at 4°C. The DNA pellet was rinsed twice with 200 µl 70% EtOH and resuspended in 100 µl TE (pH 8.0) buffer. 5 µl of the DNA, mixed with 1 µl of loading dye, was applied for mini agarose gel electrophesis to assay for altered DNA mobility. The rest of DNA was mixed with 20 µl NH_4Ac and 250 µl 100% EtOH and the solution was left for 30 minutes at room temperature. The solution was then spun for 15 minutes at 4°C. The supernatant was poured off. The DNA pellet was dried for 15 minutes under vaccum. The dried DNA pellet was resuspended in 50 µl TE buffer (pH 8.0) and left at room temperature for 20 minutes. The DNA solution was stored at -20°C freezer for future use in retransformation and sequencing.

5. DNA SEQUENCING WITH SEQUENASE VERSION 2.0

The plasmids without evidence of gross alterations were sequenced by using the dideoxyribonucleotide method, ^{35}S-labelled adenosine 5'-[α-thio] triphosphate, and the modified T7 DNA polymerase (Sequenase, United States Biochemical).

1) Preparation of templates (Alkaline Denatured):

1 μg of plasmid in 10 μl solution was mixed with 8 μl TE buffer (pH 8.0) and 2 μl of 2 N NaOH to make up a total volume of 20 μl. The mixture was left for 5 minutes at room temperature. The 20 μl of denatured plasmid was then mixed well with 4 μl of NH_4Ac (pH 4.5), 6 μl of triple distilled water, and 75 μl 100% EtOH. The mixture was left at -20°C for at least 1 hour. The template was then obtained by Eppendorf centrifugation for 15 minutes and dried under vaccum.

2) Sequencing Reactions:

The template was mixed well with 7 μl of triple distilled water, 2 μl of sequencing buffer, and 1 μl of the primer to make a final volume of 10 μl of annealing mixture. The annealing mixture was heated at 65°C for 2 minutes and then cooled slowly to below 35°C. Tubes were labeled, filled with 2.5 μl of each Termination Mixture, and then capped while cooling. Labeling Mix was diluted 5 fold with distilled water to a working concentration. Enough Sequenase Version 2.0 was also diluted 8 fold in ice cold Enzyme Dilution buffer. 4 termination tubes were prewarmed in 37°C bath. The annealed DNA mixture which was cooled to below 35°C was mixed with 1 μl of 0.1 M DTT, 2 μl of Diluted Labeling Mix, 1 μl of [^{35}S] dATP, and 2 μl of diluted Sequenase Version 2.0. The solution was mixed and incubated for 2-5 minutes at room temperature. Then 3.5 μl of the reaction solution was transfered to each termination tube (G, A, T and C) and mixed well. The reaction solution was incubated at 37°C for 2-5 minutes again. 4 μl of stop solution was added to each tube to stop the reaction. The samples

were heated to 100°C for 3 minutes immediately before loading onto a polyacrylamide gel. 2-3 µl of each of the 4 reactions were loaded onto the gel. The gel was run at 1500-2000 volts until the bromophenol blue dye reached 3 cm from the end of the gel and the top plate was removed. Water was then spread on the gel. The gel was transfered to 3 mm paper, covered with Saran Wrap, and dried in a vacuum gel dryer for 60 minutes at 80-90°C. The Saran Wrap was removed. The 3 mm paper was exposed to Kodak XAR film for 2-4 days at room temperature. The film was developed. The DNA sequence was read to check for mutations in the *supF* gene.

3) Gel Casting for a 6% Acrylamide/7 M Urea Gel:

a) Glass Plates Preparation

Two glass plates (one slightly smaller than another) were washed with detergent by using a scrub brush and wiped with 70% EtOH to dry the plates. The plastic pieces were also dried by wiping with 70% EtOH. 8.0 ml of 5% trimethylchlorositane in chloroform was spread evenly on the smaller glass plate using a paper towel to siliconize the plate. Then the plate was allowed to dry. Plastic pieces were placed on three edges of the large plate (two longer edges and one small edge). The two glass plates were clamped together by using clips. The clamped plate was placed on a gel casting stand.

b) Gel Solution Preparation

For each gel, 33.6 g urea (electrophoresis grade) was mixed with 8 ml of 10X TBE, 12 ml of 40% acrylamide, and 12 ml of 2% bis-acrylamide in a 100 ml beaker. Distilled water was used to adjust the volume to just under 80 ml and the mixture was stirred until all the urea was dissolved. The final volume of the gel solution was adjusted to 80 ml with distilled water. The gel solution was filtered into a 125 ml flask on ice using a #4 Whatman filter. 410 µl

of freshly prepared 10% ammonium persulfate and 48 µl TEMED were added to the solution before pouring.

c) Gel Pouring

The gel solution was pipeted slowly into the space between the two glass plates by using a 10 ml pipet care was taken to avoid bubbles. After the gel solution had filled the space between the plates, extra gel solution was left on the open edge. Plastic "teeth" were gently inserted into space between two plates, again avoiding any bubbles. The gel was allowed to sit at room temperature for at least 30 minutes to polymerize. The clamps were removed. The piece of plastic at the smaller, botton edge was also removed. The bottom section was rinsed with 1X TBE buffer. The gel was then set on the electrophesis apparatus and prerun for 1 hour at 1500-2000 volts or overnight at 200 volts before loading the DNA reaction samples. The wells for loading the sample were rinsed with 1X TBE buffer using a syringe before the samples were loaded.

CHAPTER III

RESULTS

A. TRANSFORMATION EFFICIENCY

The ability to transform bacterial cells to ampicillin resistance was decreased in direct proportion to the number of 4-ABP residues bound as presented in Figure 8A. About 12 4-ABP residues per plasmid were necessary to lower the transforming activity of the treated plasmid to 37% of the untreated control. This number was significantly lower than that required for dG-C8-AAF (18), dG-C8-AF (36), and dG-C8-1-A-6-NP (19) adducts to achieve the same effect (Mah et al., 1991;1989, Boldt et al., 1991), but much more than was needed for BPDE (9) and 1-NP (7) (Yang et al., 1987;1988).

B. MUTATION FREQUENCY

The treated plasmids or untreated plasmids were transfected into H293 cells and allowed 48 hours for replication. The progeny plasmids were rescued and then assayed for the frequency of *supF* mutants by transforming the SY204 indicator bacteria. There was a corresponding 4-ABP-residue dependent increase in the frequency of *supF* mutants, as shown in Figure 8B. At 55.1 adducts per pS189, the frequency was 54 times higher than the background of 0.8×10^{-4}

A total of 94 mutants from 4-ABP-modified plasmids was assayed in an agarose gel to determine the altered electrophoretic mobility (i.e. deletions or insertions > 150bp). No alteration was found in any of these 94 mutants. 77 of the mutants from 4-ABP-modified plasmids were further analyzed by DNA sequencing and the result is shown in Table 1.

Figure 8A. Decrease in the ability of the plasmid to transform bacteria to ampicillin resistance as a function of the number of adducts per plasmid (Error bars indicate the standard errors of the three determinations).

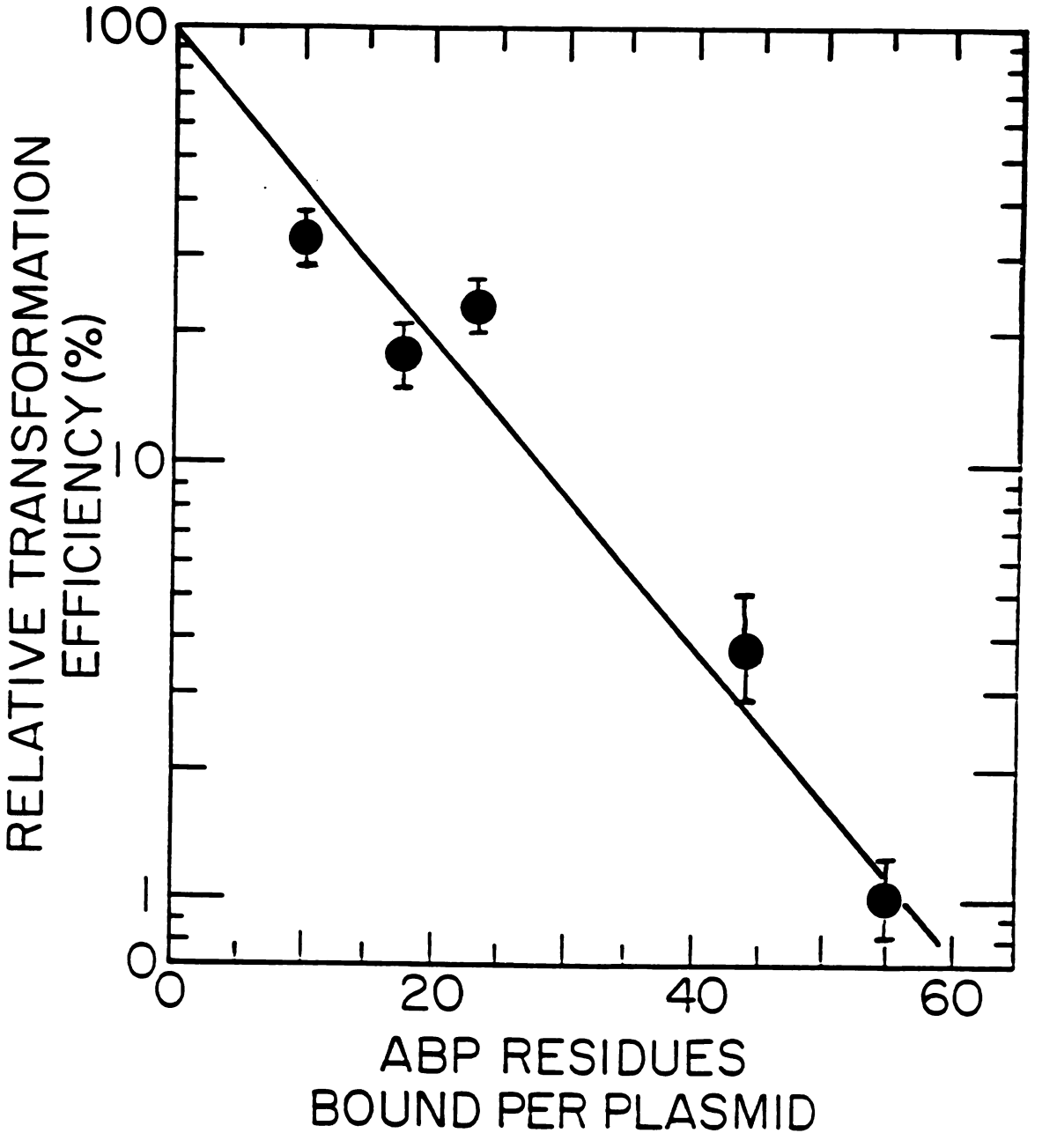

100
10
1
0
RELATIVE TRANSFORMATION EFFICIENCY (%)
0
20
40
60
ABP RESIDUES BOUND PER PLASMID

Figure 8A.

Figure 8B. Freqency of *supF* mutants as a function of the number of ABP adducts per plasmid (the error bars refer to the standard errors of the *supF* mutant frequencies obtained from a series of individual human cell transfection experiments made with each set of treated plasmid).

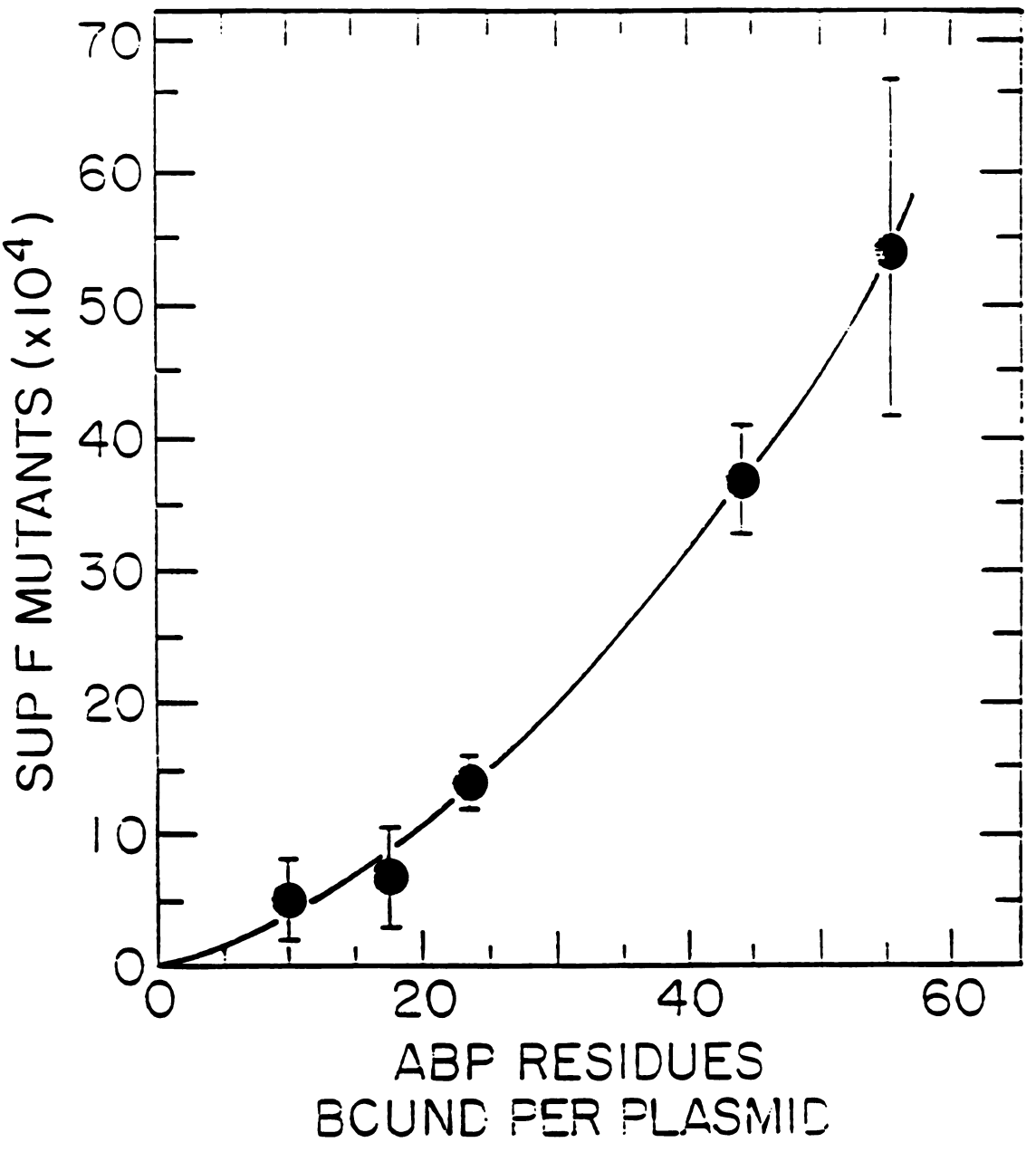

70
60
50
40
30
20
10
0
SUP F MUTANTS (x10⁴)
0
20
40
60
ABP RESIDUES
BOUND PER PLASMID

Figure 8B.

Table 1. Analysis of Mutants Obtained by Transformation of E. coli with Progeny of ABP-Modified PS189 Generated During Replication in H293 Cells

Adducts per plasmids	Number of human cell transfection experiments	Sup F mutants [a] per transformants	Frequency of sup F mutants ($x10^4$)	Plasmids with altered gel mobility [b] per No. examed	Total plasmid sup F genes sequenced	Characterization of sequenced mutants		Frequency of mutants with point mutation ($x10^4$) [e]
						Rearrangement or deletion [c]	No. with point mutations [d]	
0	13	32/390781 [f]	0.8	3/32	15	4	6	0.3
9.9	1	3/5724	5.2	0/3	3	0	3	5.2
17.7	1	3/4865	6.7	0/3	3	2	1	2.2
23.4	6	56/41264	14	0/31	23	2	21	13
44.1	6	82/22105	37	0/39	31	5	26	30
55.2	4	18/3338	54	0/18	17	2	15	48

[a] Plasmid from each mutant was assayed by a secondary transformation to determine if the inability to metabolize X-Gal resulted from inactivation of the supF gene.

[b] Alteration visible on agarose gel (>150bp).

[c] Deletion of 9 to 150 bp.

[d] Substitution, deletion, or insertion of 1, 2, or 3 bp.

[e] Calculated from fraction of mutants with point mutations times the observed frequency (column 4). The fraction of mutants with point mutations is the number in column 8 divided by that in column 6 plus those mutants showing altered gel mobility (numerator, column 5).

[f] These data for the spontaneous mutants have been published (Boldt et al., 1991).

C. KINDS OF MUTATIONS INDUCED BY 4-ABP ADDUCTS

The kinds of sequence alterations observed in unequivocally independent mutants are listed in Table 2. It can be seen from Table 2 that 86% of 4-ABP induced mutants contained 1- or 2-bp substitutions. The majority of these were single base pair substitutions. This result was similar to that from earlier studies with five structurally related residues, i.e., AAF, AF, 1-NP, 1-N-6-NOP, and BPDE (Mah et al., 1991;1989; Boldt etal., 1991; Yang et al., 1987;1988). 13% of 4-ABP-induced mutants were deletions, mainly single G·C pairs 21-100 bases apart. This also agrees with earlier studies using AAF, AF, 1-N-6-NOP, and BPDE residues (Mah et al., 1991; 1989; Boldt et al., 1991; Yang et al., 1987). However, deletions induced by 1-NP-modified plasmids in earlier studies consisted of tandem base pairs as well as those 21-100 bases apart (Yang et al., 1988). No insertions were observed among the mutations from 4-ABP-treated plasmids. But a variable, small percentage of insertions was found with the five structurally related residues (AAF, AF, 1-NP,1-N-6-NOP, and BPDE) in earlier studies (Mah et al., 1991; 1989; Boldt et al., 1991; Yang et al., 1987;1988). Only 1% of 4-ABP-modified plasmids contained a gross rearrangement. An identical result was found in both AAF and 1-N-6-NOP modified pS189 (Mah et al., 1991; Boldt et al., 1991). AF-modified pS189 showed 2% gross rearrangements (Mah et al., 1989), while no gross rearrangements were found in both 1-NP-and BPDE-modified pS189 (Yang et al., 1988;1987).

Table 3 shows the type of base pair substitutions observed in the *supF* gene of 4-ABP-modified plasmids passed through H293 cells. The major type of base pair substitution was G·C->T·A transversion. 85% of base pair substitution mutations obtained with N-AcO-TFA-ABP-treated plasmids involved G·C pairs, and 15% of them involved A·T pairs. In earlier studies with 1-NP-modified plasmids, 87% of base substitution mutations involved G·C pairs, and 13% of them involved A·T pairs (Yang et al., 1988). With BPDE-modified plasmids, 90% of the base

Table 2. Comparison of sequence alterations generated in supF by replication of carcinogen-treated or untreated plasmids in human cells

Sequence Alteration	Number of times occuring			
	Control *		ABP-Modified	
Single base substitution	7		60	
Two base substitutions				
Tandem	1		2	
< 20 bases apart	2	27%	1	86%
> 20 bases apart	0		2	
Multiple base substitutions				
(complex-50 bases apart)	0		1	
Deletions				
Single G C pair	3		4	
Single A T pair	1		0	
Tandem base pairs	0	51%	1	13%
< 20 bases	7		1	
20-100 bases	8		4	
Insertions				
Single G C pair	1		0	
Single A T pair	0	8%	0	
< 20 bases	2		0	
20-100 bases	0		0	
Gross Rearrangement	5	14%	1	1%
Total	37		77	

*These data for the control from published paper (Boldt et al., 1991).

Table 3. Comparison of the kinds of base substitutions generated in supF during replication of carcinogen-treated or untreated plasmids in human cells

Base Pair Substitution	Number of mutations observed	
	Control *	ABP-Modified
Transversions		
G C-->T A	9 (69%)	46 (64%)
G C-->C G	1 (8%)	8 (11%)
A T-->T A	0	9 (12.5%)
A T-->C G	2 (15%)	0
Transitions		
G C-->A T	1 (8%)	7 (9.7%)
A T-->G C	0	2 (2.8%)
Total	13	72

*These data for the control have been published (Boldt et al., 1991).

(G: 85% / A: 15%)

substitution mutations involved G·C pairs, and 10% of them involved A·T pairs (Yang et al., 1987). Interestingly, AF-modified plasmids did not result in any A·T base pair substitutions. In both AAF- and 1-N-6-NOP-modified plasmids, only 3% of the base pair substitution mutations involved A·T pairs (Mah et al., 1991; Boldey.al., 1991). The specific locations of the 4-ABP-induced mutations in treated plasmids and the controls are shown in Figure 9. DNA sequence analysis of 77 unequivocally independent mutants from passage of the 4-ABP-treated plasmids through the H293 cells revealed that there were 75 point mutations and that 66 out of the 77 mutants contained base substitutions. Among the 75 point mutations, 72 base substitutions were located at 28 sites of the *supF* gene, 23 base substitutions involved at G·C base pairs. However, 5 base substitutions were found to involve A·T base pairs. As noted in Table 2, the majority of mutants (60/77) contained single base substitutions. Two out of 77 mutants had tandem substitutions. One of 77 mutants had 2 base substitutions which were more than 20 bases apart. One of the 77 mutants contained a complex mutation.

The term, prominent "hot spot", was used to refer to a site in a spectrum containing 50 or more mutations where at least 8% of the mutations were located. A less prominent "hot spot" was defined as a site in a spectrum where at least 5% of such mutations were located. In the analysis of our data, one prominent "hot spot" was found at position 122 and five less prominent "hot spots" were located at positions 127, 133, 158, 160, and 169. By comparing the "hot spots" induced by the five structurally related compounds as in Table 4, it can be seen that each compound resulted in its own spectrum of mutations. Position 122 was a common prominent "hot spot" for both 4-ABP and AAF. Position 123 was a common "hot spot" for 1-NP, 1-N-6-NOP, AF, and BPDE. Position 159 was a common "hot spot" for 1-NP, 1-N-6-NOP, and AF. However, 1-N-6-NOP had its own produced prominent "hot spots" located at positions 144 and 172-176. AAF had its own prominent "hot spot" at position 155. AF had its own prominent "hot spots" at position 133 and 169. But 4-ABP and BPDE did not exhibit unique prominent "hot spots".

Figure 9. Location of independent point mutations in the coding region of the *supF* tRNA gene. The DNA strand shown is the 5' to 3' strand synthesized from the EcoRI rightward primer. The point mutations observed in the progeny of N-AcO-TFA-ABP-treated plasmids are placed below the sequence. The rectangle represents deleted nucleotide(s). The bracket indicates that it is not possible to determine which nucleotide within a run is involved in the mutation. Every 10th residue and the anticodon triplet is underlined.

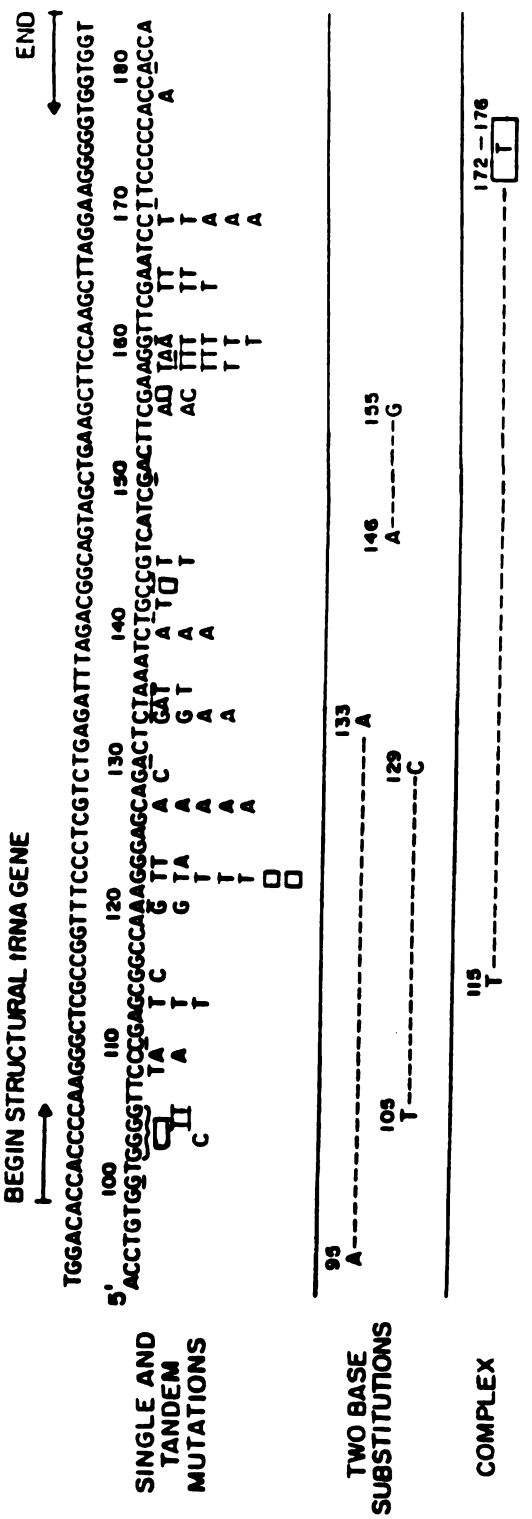

BEGIN STRUCTURAL tRNA GENE
END
TGGACACCACCCCAAGGGCTCGCCGGTTCCCTCGTCTGAGATTTAGACGGCAGTAGCTGAAGCTTCCAAGCTTAGGAAGGGGGTGGTGGT
5'
100
110
120
130
140
150
160
170
180
SINGLE AND TANDEM MUTATIONS
TWO BASE SUBSTITUTIONS
COMPLEX
95
105
115
129
133
146
155
172–176

Figure 9.

Table 4. Sites of "hot spots"[a] in the Structural Region of the sup F Gene Induced by Covalently Bound Residues of Four N-substituted Aryl Carcinogens and BPDE

1-NP	1,6-DNP	AAF	AF	BPDE	ABP
109		*[b]		109	*
	*	122	*		122
123	123		123	123	*
127	#[c]	127	*	*	#
			133	#	#
	144		*		
*		155	*	*	
159	159		159	*	
*	*		169		#
	172-176	#			*

a--A "hot spot" is defined as a site in a spectrum of at least 50 mutations, where at least 8 percent of the mutations were located.

b--The symbol, *, indicates that this particular position, although a "hot spot" for some carcinogen, is a "cold spot" for this agent. A "cold spot" is defined as a site where 2 percent or less of the observed mutations were located.

c--The symbol, #, indicates that this site, although a "hot spot" for some carcinogen, is a less prominent "hot spot" for this agent. A less prominent "hot spot" is defined as a site in a spectrum where at least 5 percent of such mutations were located.

CHAPTER IV

DISCUSSION

According to our experimental data, it could be concluded that 4-ABP was a potent inducer of point mutations in pS189 plasmids during replication in human cells, and that the mutations were induced by N-AcO-TFA-ABP-treated plasmids. This study showed that the frequency of *supF* mutants was increased with the number of adducts per plasmid as seen in Figure 8B. When the mutants obtained from the plasmids carrying 23.4, 44.1, and 55.2 adducts per plasmid, respectively, were analyzed, the results revealed that the mutant frequencies were 14, 37, and 54 times higher than that of background (0.8×10^{-4}), respectively. These plasmids also showed a low frequency of gross rearrangements or deletions and no insertions, compared to the background frequency. 91% of the 77 mutants sequenced contained point mutations, and only 9% of them involved 2 or more base pair mutations. In contrast, the background showed that only 41% of 37 mutants sequenced were related to point mutations and that 59% of them were involved with 2 or more base pair mutations as shown in Table 2. The fact that 85% (61/72) of the base substitutions and 100% (4/4) of the single base pair deletions by 4-ABP adducts occurred at G·C base pairs strongly suggested that mutagenesis was targeted to sites where adducts occurred, because N-AcO-TFA-ABP formed adducts predominantly (95%) with guanine as seen in Figure 4. 15% (11/72) of the base substitutions involved A·T base pairs. Among these A·T base pair substitutions, 2 out of 11 were observed in mutants derived from plasmids treated with a low dose carcinogen (9.9 adducts per plasmid) and 4 of them were derived from a high dose (23.4 adducts per plasmid). The remaining 5 A·T base pairs were also from plasmids exposed to a high dose of carcinogen (55.2 adducts per plasmid). Although N-AcO-TFA-ABP formed minor adducts with adenine (5%), as seen in Figure 4, 82% (9/11) of the base substitutions involving A·T pairs were from high dose of carcinogen.

This result suggests that the mutagenesis was targeted to sites where adducts occurred. The base substitution mutations that occurred at both G·C and A·T base pair targets were also observed in *E. coli* virus M13mp10 that carried 4-ABP adducts after replicating in a SOS-processing environment (Lasko et al., 1988). Therefore, it can be concluded that the N-AcO-TFA-ABP-induced base substitutions were caused by 4-ABP adducts, rather than the result of apurinic sites (Loeb et al., 1986). This conclusion was supported by earlier studies in our laboratory with data from BPDE, 1-NOP, 1-N-6-NOP, N-AcO-TFA-AAF, and N-AcO-TFA-AF-treated plasmids (Yang et al., 1987; 1988; Boldt et al., 1991; Mah et al., 1991 ;1988).

The majority (64%) of base substitutions observed were G·C ->T·A transversions. Several mechanisms have been proposed to explain this observation. Loeb et. al., (1986) suggested that such transversion mutations could result from apurinic sites in DNA when DNA polymerase inserted adenosine triphophate into the opposite strand during replication. Since dG-C8-ABP adducts were shown as the major, stable adducts (Beland et al., 1983;1985), this kind of transversion mutation is less likely to have been caused by depurination. However, such a mutation could be caused by the action of repairing enzyme inside the human cell. A second explanation, suggested by Strauss et al., (1982) is that the DNA polymerase preferentially inserts an adenine nucleotide across a non-instructional base containing a bulky adduct ("A role"). A third explanation is that by Eisenstadt et al. (1982) who suggested that changes in the conformation of guanine by the adduct might allow stable purine·purine mispairing, and that this mispairing might then go undetected.

HPLC analysis of our modified pS189 showed that 95% of the adducts were dG-C8-ABP and 5% were dA-C8-ABP. No dG-N2-ABP adducts were found, as shown in Figure 4. However, our mutation data indicated that 85% of the base substitutions occurred at G·C base pairs and 15% of the base substitutions occurred at A·T base pairs. The G·C base pair substitutions mainly consisted of G·C->T·A

transversions, while A·T base pairs mainly consisted of A·T->T·A transversion. 9 out of the 11 base substitutions involving A·T base pairs were derived from plasmids carrying relatively high levels of carcinogen residues (23.4 or 55.2 adducts per plasmid). This suggests that such base substitution mutations were caused by 4-ABP, rather than being background mutations. As noted in Figure 9, these base substitutions which occurred at A·T base pairs were not random. Instead they were related to a certain sequence such as 5'CAAAG3', 5'CTAAATC3', 5'GAAGG3', and 5'GAATCC3'. This observation indicates that at least two adenines have to be adjacent to each other. The two adjacent adenines on these particular sequences could easily be targeted by 4-ABP. Potentially mutagenic lesions at these sites may be less favorable for the repair enzyme to induce mutation. A model by Topal and Fresco (1976) indicated that purine residues were in the syn-configuration instead of the normal anti-configuration and that purine residues paired specifically with the rare tautomeric iminoform of adenine or guanine. Is so, this pairing would be expected to yield G·C->T·A transversions, A·T->T·A transversions, and G·C->C·G transversions.. The assumption that adenine adducts were the premutagenic lesion is strengthened by the fact that the base substitutions that occurred at A·T base pairs were predominantly A·T->T·A transversion. This is what would be predicted by the "A role". *E. coli* virus M13mp10 carrying 4-ABP adducts was studied in a SOS-processing environment. 4-ABP lesions accounted for 70% of dG-C8-ABP and 20% of dA-C8-ABP and dG-N2-ABP combining together. G·C base pairs were the major targets for base pair substitution mutation. Certain A·T base pairs were also involved in significant mutagenic activity. In the prokaryotic repair system, unlike the human cell system, G·C->T·A transversions were not the majority of base substitutions. This suggests that the prokaryotic repair system might differ from the eukaryotic system. A "hot spot" was observed at A·T base pairs adjacent to a run of 5Gs, predominantly A·T->T·A transversion (Lasko et al., 1988). A less prominent "hot spot" was detected at A·T base pairs adjacent to a run of 2Gs (position 158) in the human cell system, as shown in Figure 9. There were 13%

deletions and 86% base substitutions. Among the deletions, the majority was single G·C base pairs and 20-100 base pairs. Three out of four single G·C base pair deletions were found to be located within a run of 2-3 Gs. One tandem base deletion was located in a run of 4 Gs, as seen in Figure 9. 4-ABP lesions caused a "hot spot" that involved the deletion of G·C or C·G within the C·G octamer. This accounts for 82-94% of induced revertants in bacterial strain TA98 and 99.5% of induced revertants in TA1538 strain of Salmonella typhimurium (Levin et al., 1991). These results further suggest that neighboring sequences may determine the spectrum of mutagenesis.

Although adducts formed by all six structurally related chemical carcinogens (N-AcO-TFA-4-ABP, N-AcO-TFA-AAF, N-AcO-TFA-AF, 1-N-6-NOP, 1-NP, and BPDE) induced point mutations, with single base substitutions being predominant and with G·C->T·A transversions being the most common mutation, the spectra of mutations in the *supF* tRNA gene induced by different carcinogens differed significantly from one another. 4-ABP exhibited one prominent "hot spot" (position 122), which is in common with AAF. But this "hot spot" was a "cold spot" for 1-NP, 1-N-6-NOP as well as AF (Yang et al., 1988; Boldt et al., 1991; Mah et al., 1989). The prominent "hot spot" for 1-N-6-NOP at position 172-176 was a "cold spot" for 4-ABP (Boldt et al., 1991). The "hot spots" for 4-ABP and AAF were at the first G in a run of three Gs (position 122). However, the "hot spots" for mutations induced by 1-NP, 1-N-6-NOP, AF, and BPDE were all at the middle G in a run of three Gs (position 123). Also, four Gs in a row 102-105 were more sensitive to mutations induced by 4-ABP adducts than by adducts formed by the other five structurally-related carcinogens (Yang et al., 1987;1988; Boldt et al., 1991; Mah et al., 1989;1991). This difference may reflect the fact that the ability of structurally-related carcinogen adducts to alter DNA structure may differ at specific sites. DNA repair may also play an important role in determining the susceptibility of particular sites to mutagenesis.

REFERENCES

Bartsch, H., Caporaso, N., Coda, M., Kadlubar, F., Malaveille, C., Skipp P., Talaska, G., Tannenbaum, S. R., Vineis, P. (1990) Carcinogen hemoglobin adducts, urinary mutagenicity, and metabolic phenotype in active and passive cigarette smokers. *J. Natl. Cancer Inst.*, **82**, 1826-1831.

Bean, C. L., Armstrong, M. A., and Galloway, S. M. (1991) Effect of sampling time on chromosome aberration yield for seven chemicals in Chinese hamster ovary cells. *EMS Abstracts*, **10**, 20.

Beland, F. A., Beranek, D. V., Dooley, K. L., Heflich, R. H., and Kadlubar, F. F. (1983) Arylamine-DNA adducts *in vitro* and *in vivo* : Their role in bacteria mutagenesis and urinary bladder carcinogenesis. *Environ. Health Perspect*, **49**, 125-134.

Beland, F. A. and Kadlubar, F. F. (1985) Formation and persistence of arylamine DNA adducts *in vivo* . *Environ. Health Perspect*, **62**, 19-30.

Block, N. L., Sigel, M. M., Lynne, C. M., Ng, A. B., and Grosberg, R. A. (1978) The initiation, progress, and diagnosis of dog bladder cancer induced by 4-aminobiphenyl. *Invest. Urol.*, **16**, 50-54.

Boldt, J., Mah, M.C-M., Wang, Y. C., Smith, B. A., Beland, F. A., Maher, V. M., and McCormick, J. J. (1991) Kinds of mutations found when a shuttle vector containing adducts of 1,6-dinitropyrene replicates in human cells. *Carcinogenesis*, **12**, 119-126.

Bonser, G. M., Clayson, D. B., and Jull, J. W. (1956) The induction of tumours of the subcutaneous tissues, liver and

intestine in the mouse by certain dyestuffs and their intermediates. *Br. J. Cancer*, **10**, 653-667.

Broyde, S., Hingerty, B. E., and Srinivasan, A. R. (1985) Influence of the carcinogen 4-aminobiphenyl on DNA conformation. *Carcinogenesis*, **6**, 719-725.

Calos, M. P., Lebkowski, J. S., Botchan, M. R. (1983) High mutation frequency in DNA transfected into Mammalian cells. *Proc. Natl. Acad. Sci. USA.*, **80**, 3015-3019.

Clayson, D. B. (1962) *Chemical Carcinogenes Little*, Brown, Boston.

Clayson, D. B., and Garner, R. C. (1976) Carcinogenic aromatic amines and related compounds. In: *Chemical Carcinogens*. ACS Monograph, **173**, C. E. Searle (Ed.) American Chemical Society, Washington, D. C., pp. 366-461.

Coghlin, J., Gann, P. H., Hammond, S. K., Skipper, P. L., Taghizadeh, K, Paul, M., Tannenbaum, S. R. (1991) 4-Aminobiphenyl hemoglobin adducts in fetuses exposed to the tobacco smoke carcinogen in Utero. *J. Natl. Cancer Inst.*, **83**, 274-280.

Eisenstadt, E., Warren, A. J., Porter, J., Atkins, D., and Miller, J. H. (1982) Carcinogenic expoxides of benzo[a]pyrene and cyclopental [cd]pyrene induce base substitutions via specific transversions. *Proc. Nat. Acad. Sci. USA*, **79**,1945-1949.

Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.*, **36**, 59-72.

Hueper, W. C., Wiley, F. H., and Wolfe, H. D., (1938) Experimental production of bladder tumors in dogs by

administration of Beta-Naphthylamine. *J. Ind. Hyg. Toxicol.*, **20**,46-84.

Hueper, W. C., (1969) *Occupational and Environmental Cancers of the Urinary System.* Yale University Press, New Haven, Conn.

IARC Monographs, **1**, 74-79 (1972).

IARC Monographs, Suppl. **6**, 60-63, 1987.

IARC Monographs, Suppl. **7**, 92, 1988.

Kadlubar, F. F., Miller, J. A., and Miller, E. C. (1977) Hepatic microsomal glucuronidation and nucleic acid binding of N-hydroxy arylamines in relation to urinary bladder carcinogenesis. *Cancer Res.*, **37**, 805-814.

Kinosita, R., (1937) Studies on the cancerogenic chemical substances. *Japan. Pathol. Soc. Trans.*, **27**, 665-727.

Kraemer, K. H. and Seidman, M. M. (1989), Use of *supF*, an *Escherichia coli* tyrosine suppressor tRNA gene, as a mutagenic target in shuttle-vector plasmids. *Mutat. Res.*, **220**, 61-72.

Lasko, D. D., Harvey, S. C., Malaikal, S. B., Kadlubar, F. F., and Essigmann, J. M. (1988), Specificity of mutagenesis by 4-aminobiphenyl. *J. of Biol. Chem.*, **263**, 15429-15435.

Lebkowski, J. S., Clancy, S., Miller, J. H., and Calos, M. P. (1985) The lacI shuttle: Rapid analysis of the mutagenic specificity of ultraviolet light in human cells. *Proc. Natl. Acad. Sci. USA*, **82**, 8606-8610.

Lebkowski, J. S., Dubridge, R. B., Antell, E. A., Greisen, K. S., Calos, M. P. (1984) Transfected DNA is mutated in monkey, mouse, and human cells. *Mol. and Cell. Bio.*, **4**, 1951-1960.

Leichtenstern, O. (1898) Uber Harnblasenentzundung and Harnblasengeschwulste ber Arberitern in Farbfabriken, *Dtsch. Med. Wochenschr.*, **24**, 709-713.

Levin, J. G., Schaper, R. M., and DeMarini, D. M., Spectra of mutations induced by 4-aminobiphenyl and cigaratte smoke condensates at the his D3052 allele of *Salmonella typhimurium* strains TA98 and TA1538. unpublished paper.

Loeb, L. A. and Preston, B. D. (1986) Mutagenesis by apurinic/apyrimidinic sites. *Annu. Rev. Genet.*, **20**, 201-230.

Maclure, M., Katz, R. B., Bryant, M. S., Skipper, P. L., Tannenbaum, S. R. (1989) Elevated blood levels of carcinogens in passive smokers. *Am. J. Public Health*, **79**, 1381-1384.

Mah, M.C-M., Boldt, J., Culp, S. J., Maher, V. M., and McCormick, J. J. (1991) Replication of acetylaminofluorene-adducted plasmids in human cells: Spectrum of base substitutions and evidence of excision repair. *Proc. Nat. Acad. Sci. USA*, **88**, 10193-10197.

Mah, M.C-M., Maher, V. M., Thomas, H., Reid, T. M., King, C. M., and McCormick, J. J. (1989) Mutations induced by aminofluorene-DNA adducts during replication in human cells. *Carcinogenesis*, **10**, 2321- 2328.

Maher, V. M., Miller, E. C., Miller, J. A., and Szybalski, W. (1968) Mutations and decreases in density of transforming DNA produced by derivatives of the carcinogens 2-acetyl-aminofluorene and N-methyl-4-aminoazobenzene. *Mol. Pharmacol.*, **4**, 411-426.

Maher,V. M., Miller, E. C., Miller, J. A., and Summers, W. C. (1970) Mutation and loss of transforming activity of *Bacillus subtilis* DNA after reaction with esters of carcinogenic N-hydrox aromatic amides. *Cancer Res* ., **30**: 1473-1480.

Maniatis, T., Fritsch, E. F., and Sambrook, J, (1982) In: *Molecular Cloning*: A Laboratory Manual, p. 86-94. Cold Spring Harbor laboratory, Cold Spring Harbor, N.Y.

McCann, J., Choi, E., Yamasaki, E., and Ames, B. N. (1975) Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals. *Proc. Nat. Acad. Sci. USA*, **72**, 5135-5139.

Melick, W. F., Escue, H. M., Naryka, J. J., Mezera, R. A., and Wheeler, E. P. (1955) The first reported cases of human bladder tumors due to a new carcinogen-xenylamine. *The J. of Urol.*, **74**, 760-766.

Melick, W. F., Naryka, J. J., and Kelly, R. E. (1971) Bladder c ancer due to exposure to Para-Aminobiphenyl: A 17-year followup. *The J. of Urol.*, **106**, 220-226.

Miller, E. C., Miller, J. A., and Hartmann, H. A. (1961) N-hydroxy-2-acetylaminofluorene: a metabolite of 2-acetylaminofluorene with increased carcinogenic activity in the rat. *Cancer Res.*, **21**, 815-824.

Miller, E. C., Miller, J. A., and Enomoto, M. (1964) The comparative carcinogencities of 2-acetylaminofluorene and its N-hydroxymetabolites in mice, hamsters, and guinea pigs. *Cancer Res.*, **24**, 2018-2032.

Miller, J. A., and Miller, E. C. (1967) Activation of carcinogenic aromatic amines and amides by N-hydroxylation *in vivo*. In *Carcinogenesis: A Broad Critique*. M.D. Anderson Tumor

Institute, University of Texas. Williams and Wilkins, Baltimore, pp. 397-420.

Pai, V., Boomfield, S. F., and Gorrod, J. W. (1985) Mutagenicity of N-hydroxylamines and N-hydroxycarbamates towards strains of Escherichia coli and Salmonella typhimurium. *Mutat. Res.*, **151**, 201-207.

Razzaque, A., Chakrabarti, S., Joffee, S., and Seidman, M. (1984) Mutagenesis of a shuttle vector plasmid in mammalian cells. *Mol. and Cell. Bio.*, **4**, 435-441.

Rehn, L. (1895) Blasengeschwulste bei Fuchsin-Arbeitern. *Arch. Klin. Chir.*, **50**, 588-600.

Ronco, G., Vineis, P., Bryant, M. S., Skipper, P. L., Tannenbaum, S. R. (1990) Hemoglobin adducts formed by aromatic amines in smokers: sources of inter-individual variability. *Br. J. Cancer*, **61**, 534-537.

Sarkar, S., Dasgupta, U. B., and Summers, W. C. (1984) Error-prone mutagenesis detected in mammalian cells by a shuttle vector containing the *supF* gene of Escherichia coli. *Mol.Cell. Biol.*, **4**, 2227-2230.

Sasaki, T., and Yoshida, T. (1935) Experimentelle Erzeugung des Lebercarcinoms durch Futterung mit O-Amidoazotoluol. *Virchow's Arch Pathol Anat.*, **295**, 175-200.

Schmidt, M. B. (1924) Uber vital Fettfarbung in Geweben und Sekreten durch Sudan geschwulstartige Wucherungen derausscheidenden Drusen. Virchow's Arch. Pathol. Anat., **253**, 432-451.

Schieferstein, G. J., Littlefield, N. A., Gaylor, D. W., Sheldon, W. G. & Burger, G. T. (1985) Carcinogenesis of 4-aminobiphenyl in

BALB/cStCrlfC3Hf/Nctr mice. *Eur. J. Cancer Clin. Oncol.*, **21**, 865- 873.

Seidman, M. M. (1989) The development of transient SV 40 based shuttle vectors for mutagenesis studies: Problems and solutions. *Mutat. Res.*, **220**, 55-60.

Seidman, M. M., Dixon, K., Razzaque, A., Zagursky, R. J., and Berman, M. L. (1985) A shuttle vector plasmid for studying carcinogen-induced point mutations in mammalian cells. *Gene.*, **38**, 233-237.

Strauss, B., Rabkin, S., Sagher, D., and Moore, P. (1982) The role of DNA polymerase in base substitution mutagenesis on noninstructional templates. *Biochimie*, **64**, 829-838.

Tamura, N. and King, C. M. (1990) Comparative survival of aminobiphenyl-and aminofluorene-substituted plasmid DNA in Escherichia coli Uvr endonuclease deficient strains. *Carcinogenesis*, **11**, 535-540

Tomatis, L., Agthe, C., Bartsch, H., Huff, J.,Montesano, R., Saracci, R., Walker, E., and Wilbourn, J. (1978) Evaluation of the carcinogenicity of chemicals: A review of the Monograph Program of the International Agency for Research on Cancer. *Cancer Res.*, **38**, 877-885.

Topal, M. D. and Fresco, J. R. (1976) Complementary base pairing and the origin of substitution mutations. *Nature*, **263**, 285- 289.

Vineis, P., Caporaso, N., Tannenbaum, S. R., Skipper, P. L., Glogowski, J., Bartsch, H., Coda, M., Talaska, G., Kadlubar, F., (1990) Acetylation phenotype, carcinogen-hemoglobin adducts, and cigaratte smoking. *Cancer Res.*, **50**, 3002-3004.

Walpole, A. L., Williams, M. H., and Roberts, D. C. (1954) Tumours of urinary bladder in dogs after ingestion of 4-aminodiphenyl. *Br. J. Ind. Med.*, **11**, 105-109.

Wilson, R. H., DeEds, F., & Cox., A. J. Jr.(1941) The toxicity and carcinogenic activity of 2-acetaminofluorene. *Cancer Res.*, **1**, 595-608.

Yang, J. L., Maher, V. M., and McCormick, J. J. (1987) Kinds of mutations formed when a shuttle vector containing adducts of (±)-7b,8a- dihydroxy-9a,10a-epoxy-7,8,9,10-tetrahydrobenzo-[a]pyrene replicates in human cells. *Proc. Nat. Acad. Sci. USA*, **84**, 3787-3791.

Yang, J. L., Maher, V. M., and McCormick, J. J. (1988) Kinds and spectrum of mutations induced by 1-Nitrosopyrene adducts d during plasmid replication in human cells. *Mol. and Cell. Biol.*, **8**, 3364-3372.

Yoshida, T. (1933) Uber die serienweise Verfolgung der Veranderungen der Leber der experimentellen Hepatomerzeugung durch O-Aminoazotoluol. *Trans. Japan Pathol. Soc.*, **23**, 636-638.

Zagursky, R. J., Baumeister, K., Lomax, N., and Berman, M. L. (1985) Rapid and easy sequencing of large linear double-stranded DNA and supercoiled plasmid DNA. *Gene Anal.Technol.*, **2**, 89-94