SPECTRA OF MUTATIONS INDUCED BY AMINOFLUORENE, ACETYLAMINOFLUORENE AND STRUCTURALLY RELATED CARCINOGEN ADDUCTS DURING SHUTTLE VECTOR REPLICATION IN HUMAN CELLS

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ABSTRACT

SPECTRA OF MUTATIONS INDUCED BY AMINOFLUORENE, ACETYLAMINOFLUORENE AND STRUCTURALLY-RELATED CARCINOGEN ADDUCTS DURING SHUTTLE VECTOR REPLICATION IN HUMAN CELLS

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2-Acetylaminofluorene (AAF), a well studied liver carcinogen, forms two major DNA adducts, viz., *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-C8-AAF), and its deacetylated derivative *N*-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF). These adducts have been shown to induce different degrees of conformational alterations in the DNA, and to be removed from DNA at different rates. Studies of the kinds of mutations induced when plasmids randomly modified with these two adducts were allowed to replicate in bacteria showed that dG-C8-AF adducts caused primarily base substitutions, mainly G·C --> T·A transversions, whereas dG-C8-AAF adducts almost exclusively induced frameshifts. To investigate the mechanisms of mutagenesis by reactive derivatives of AAF in mammalian cells, I determined the frequency and kinds of mutations induced when a shuttle vector containing dG-C8-AF or dG-C8-AAF adducts replicated in human cells. Using the same assay, I also determined the spectrum of

mutations induced by 1,6-dinitropyrene (1,6-DNP) residues which also bind to the C-8 position of guanine.

For this purpose, a shuttle vector pZ189 or pS189, carrying a bacterial suppressor tRNA (*supF*) gene as the target for mutations, was treated with radiolabeled carcinogens to obtain AF, AAF and 1,6-DNP residues covalently bound to the DNA. These plasmids were introduced into human cells and allowed to replicate. The progeny plasmids were examined for the frequency of *supF* mutants and the kinds of mutations and their location in the gene. The frequency of mutants increased with dose and number of adducts. When compared on the basis of the number of adducts per plasmid, AAF and 1,6-DNP residues were equally mutagenic; and they were about two times more mutagenic than AF residues. All three carcinogens induced mainly base substitutions (about 80%), predominantly G·C -->T·A transversions. However, AAF and 1,6-DNP residues induced a high frequency of minus one frameshifts. The mutations were not randomly distributed and some mutational "hot spots" were shared by two or more carcinogens. However, each agent induced its unique spectrum of mutations. A DNA polymerase termination assay used to estimate the sites of carcinogen binding showed a good correlation between the sites of adduct formation and the sites of mutation induction.

This work is dedicated to

my husband,	Everson
my parents,	Ma Chi Liang Ma Hwang Yu
my countries,	Brazil Taiwan

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ABBREVIATIONS

AF	2-aminofluorene
AAF	2-acetylaminofluorene
(-)-BcPhDE-2	benzo[*c*]phenanthrene(4*R*,3*S*)-dihydrodiol(2*S*,1*R*)-epoxide
bp	base pair
BPDE	(±)-7,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene
CHO	Chinese hamster ovary cell line
COS	simian kidney cell line
dG-C8-AAF	*N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene, also reffered as AAF adduct
dG-C8-AF	*N*-(deoxyguanosin-8-yl)-2-aminofluorene, also reffered to as AF adduct
dG-C8-1-A-6-NP	*N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene
dG-N^2-AAF	3-(deoxyguanosin-N^2-yl)-2-acetylaminofluorene
dhfr	dihydrofolate reductase gene
DNP	dinitropyrene
1,6-DNP	1,6-dinitropyrene
HPLC	high-pressure liquid chromatography
lacI	a gene coding for the repressor of the lactose operon
MAB	*N*-methyl-4-aminoazobenzene
N-AcO-AAF	*N*-acetoxy-2-acetylaminofluorene
N-AcO-AF	*N*-acetoxy-2-aminofluorene
N-AcO-TFA-AF	*N*-acetoxy-*N*-trifluoroacetyl-2-aminofluorene
N-OH-AAF	*N*-hydroxy-2-acetylaminofluorene

N-OH-AF	*N*-hydroxy-2-aminofluorene
1-N-6-NOP	1-nitro-6-nitrosopyrene
1-NOP	1-nitrosopyrene
1-NP	1-nitropyrene
supF	a gene coding for a tyrosine suppressor tRNA
SV40	simian virus 40
TFA	trifluoroacetic acid
nPAH	nitrated polycyclic aromatic hydrocarbons

INTRODUCTION

The aromatic amine 2-acetylaminofluorene (AAF) is a well known liver carcinogen, and is one of the most studied model aromatic compounds used to investigate the molecular mechanisms of carcinogenesis and mutagenesis. This strong hepatic carcinogen has been shown to induce tumors in diverse organs in experimental animals (Wilson et al., 1941; Miller, 1978; Garner et al., 1984). However, AAF *per se* is not carcinogenic (Miller et al., 1961; 1962) or mutagenic (Maher et al., 1968; McCann et al., 1975), but must undergo conversion to reactive metabolites which can bind to macromolecules of the target tissues (Miller & Miller, 1966; 1969a; 1969b; Miller, 1978). The metabolic derivatives of AAF have been shown to bind to cellular proteins (Miller & Miller, 1947) and nucleic acids (Kriek, 1965; Kriek et al., 1967; Williard & Irving, 1964).

When reactive metabolites of AAF interact with DNA, two major adducts at the C-8 position of guanine are formed : *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-C8-AAF) and its deacetylated form, *N*-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF) (Kriek, 1965; Kriek et al., 1967; Westra & Visser, 1979). The latter comprises about 80% of the total adducts, whereas the former represent 15% of the adducts formed (Irving & Veazey, 1969; Kriek, 1972; Poirier et al., 1982). A minor adduct which binds to the N^2 position of guanine has also been found and comprises 5% of the total adducts (Westra et al., 1976). The two major adducts have

been shown to cause different degrees of conformational changes in the DNA helix (Grunberger et al., 1970; Fuchs, 1975; Santella et al., 1980; Broyde & Hingerty, 1983). This difference seems to have certain effects on the biological properties of dG-C8-AF and dG-C8-AAF adducts, such as the rate of repair (Kriek, 1972; Howard et al., 1981; Poirier et al., 1982) and the kinds of mutations induced by these adducts in prokaryotic cells (Koffel-Schwartz et al., 1984; Bichara & Fuchs, 1985). The mutagenic action of the reactive metabolic derivatives of AAF in bacteria (Beranek et al., 1982), human cells (Maher & Wessel, 1975; Aust et al., 1984) and other mammalian cells (Carothers et al., 1986; 1989; Heflich et al., 1988) is well documented. The specific kinds of mutations induced by dG-C8-AF or dG-C8-AAF adducts in bacteria have been extensively studied using plasmids or bacteriophages that were modified with one or other of these two residues, either randomly (Koffel-Schwartz et al., 1984; Bichara & Fuchs, 1985; Gupta et al., 1988) or at specific sites (Romano et al., 1987; Moriya et al., 1988; Gupta et al., 1989; Koehl et al., 1989). Fuchs and coworkers using pBR322 plasmid which was randomly modified by either AF or AAF residues showed that AF induced primarily base substitutions, whereas AAF induced >90% frameshifts when the plasmid was allowed to be replicated in *E. coli* (Koffel-Schwartz et al., 1984; Bichara & Fuchs, 1985). The results of the studies using site-specific adducts were sometimes controversial, probably because the sequence context is important for the mutagenic effects. Only in the past five years have a few papers been published on the specific kinds of mutations induced by aminofluorene (AF) and the acetylated AAF adducts in mammalian systems. Gentil et al. (1986) and Moriya et al.(1988) used simian virus 40 (SV40) vector and shuttle vector

respectively to study AAF induced mutations in monkey kidney cells. Carothers et al. (1986; 1989) taking advantages of the polymerase chain reaction technique, studied the kinds of mutations induced by *N*-acetoxy-2-acetylaminofluorene (N-AcO-AAF) in the endogenous dihydrofolate reductase gene of Chinese hamster ovary (CHO) cells. The latter study showed that N-AcO-AAF, which forms about 90% of dG-C8-AF adducts when CHO cells are exposed to this carcinogen, induced significant numbers of putative point mutations (72%). 90% of the point mutations that were analyzed by DNA sequencing showed base substitutions, mainly G•C --> T•A transversions.

The determination of the specific kinds and locations of mutations induced by AF and AAF adducts in mammalian cells can contribute to the understanding of the molecular mechanisms of mutagenesis by AAF in these cells. However, comparative studies of the spectrum of mutations induced by AF and AAF adducts in mammalian systems have not been reported. One of the reasons is that when metabolic derivatives of AAF react with DNA *in vivo* or with mammalian cells in culture, dG-C8-AF constitutes 80% or more of the total adducts formed (Irving & Veazey, 1969; Allaben et al., 1983; Poirier et al., 1980; Carothers et al., 1986; Heflich et al., 1988), making it difficult to study the mutations induced by AAF adducts in mammalian cells. Another obstacle to such comparative studies is the difficulty in obtaining dG-C8-AF adducts. This deacetylated product can be formed *in vitro* by treatment of DNA with *N*-hydroxy-aminofluorene at pH 5 or by alkaline deacetylation of dG-C8-AAF. However, the AF-modified DNA obtained by these methods sometimes is unstable and decomposes rapidly in aqueous solution (Singer & Grunberger, 1983).

A novel method to study the spectrum of mutations induced by AF and AAF adducts in a human cell system became plausible with the development of shuttle vectors, i.e., plasmids carrying a defined target gene that can replicate both in mammalian cells and in bacteria (Calos et al., 1983; Razzaque et al., 1983; Lebkowski et al., 1985; Seidman et al., 1985), and the synthesis of *N*-acetyl-*N*-trifluoroacetyl-2-aminofluorene (N-AcO-TFA-AF) by King and his group (Johnson et al., 1986). When reacted with DNA *in vitro* at pH 7 this trifluoro derivative of N-AcO-AAF almost exclusively forms dG-C8-AF adducts that are chemically stable (Johnson et al., 1986; 1987).

The objectives of this present work were (1) to investigate at the sequence level the specific kinds of mutations induced by AF and AAF adducts 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 and to see if these two adducts induce the same mutational spectrum; (3) to determine the relative frequency of the adduct formation at different sites within the DNA sequence of the target gene and to see if it correlates with the frequency of mutation induction at these sites; (4) to investigate whether these two adducts have similar biological effectiveness, i.e., to compare their ability to interfere with bacterial transformation and to induce mutations during shuttle vector replication in human cells; (5) to compare the spectra of mutations induced by AF and AAF adducts in the *supF* gene with the spectrum of mutations induced by 1,6-dinitropyrene (1,6-DNP) adducts in the same assay. The latter study has been a collaborative work with my colleague Janet Boldt. Comparasons can also be made with the spectra of mutations induced by two other

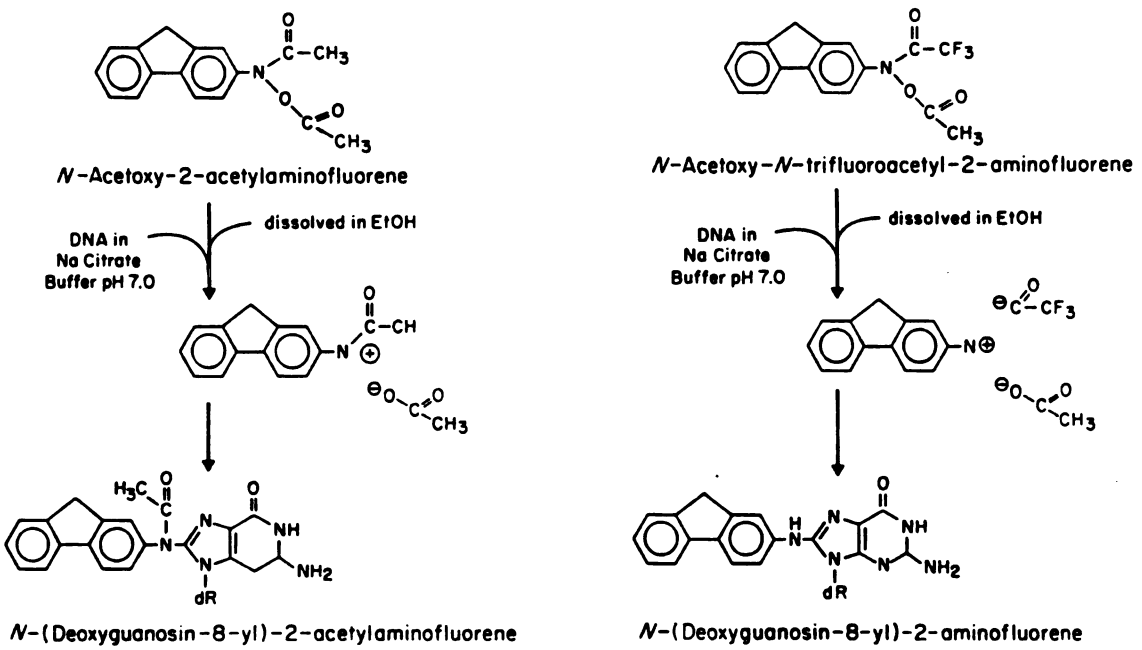

Figure 1. ***In vitro*** **interaction of *N*-acetoxy-2-acetylaminofluorene or *N*-acetoxy-*N*-trifluoroacetyl-2-aminofluorene with DNA resulting in *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene or its deacetylated derivative *N*-(deoxyguanosin-8-yl)-2-aminofluorene, respectively.**

stucturally-related carcinogens, viz., 1-nitropyrene and (±)-7β,8α-dihydroxy-9α, 10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene in this same gene, which have been previously studied in this laboratory (Yang et al., 1987; 1988). It is now generally recognized that mutations are involved in the multistep carcinogenesis process by causing one or more changes required for the process (Zarbl et al., 1985; Baker et al., 1989; Horowitz et al., 1989). By determining whether structurally-related chemical carcinogens cause similar spectra of mutations in human cell systems, we might get insight into mechanisms by which these carcinogens cause mutations.

Chapter I reviews the background literature on AAF mutagenesis. Chapter II consists of a manuscript published in the December 1989 issue of *Carcinogenesis*, 10, 2321-2328. It describes the research I carried out to determine the specific kinds of mutations induced when shuttle vector pZ189 containing AF adducts is replicated in human 293 cells, as well as their location in the *supF* gene. The N-AcO-TFA-AF used to obtain dG-C8-AF adducts in this study was synthesized by Dr. Thomas M. Reid and Dr. Charles M. King from Michigan Cancer Foundation and kindly donated to us. The treatment of the plasmids, human cell transfection and bacterial transformation experiments, as well as the isolation, agarose gel electrophoresis and DNA sequence analysis of the mutants were performed by me. I also optimized the conditions for the *in vitro* polymerization termination assay and carried out these experiments. Dr. Harvey Thomas, who was a research associate in this laboratory at that time participated in the amplification of 30% of the mutants that were sequenced. Chapter III describes comparable work I carried out with shuttle vector pZ189

containing AAF adducts induced by N-AcO-AAF and compares the results obtained with AAF adducts with those I found with AF adducts. The contribution of Dr. Janet Boldt to this work consisted of performing DNA sequencing of 40% of the mutants. The format used for Chapter III is that of a manuscript to be submitted to the *Journal of Molecular Biology* or the *Proceedings of National Academy of Science*. Chapter IV, which consists of a manuscript published in the January 1991 issue of *Carcinogenesis*, 12, 119-126, describes the work I did in collaboration with my colleague Dr. Boldt on the spectrum of mutations induced when shuttle vector pS189 was treated with 1-nitro-6-nitrosopyrene (1-N-6-NOP) and allowed to replicate in human 293 cells. Dr. Frederick A. Beland and Dr. Beverly A. Smith synthesized the tritium labeled 1-N-6-NOP compound used to obtain 1,6-DNP adducts in this study and conducted the ^{32}P-postlabeling analysis of the adducts. I treated the plasmid with this carcinogen, determined the amount of adducts formed, and the degree of interference with bacterial transformation and performed the human cell transfection experiments. The research involved in the bacterial transformation experiments, and the isolation and agarose gel electrophoresis analysis of the mutants obtained was carried out in equal share by Boldt, Wang and me. DNA sequence analysis of the mutants was done 70% by Boldt and 30% by me.

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CHAPTER I

LITERATURE REVIEW

A. Importance of polycyclic aromatic hydrocarbons and aromatic amines in the history of chemical carcinogenesis

References to occupational cancer can be found as early as the 16th century, as cited by Searle & Teale (1990). However, the first clear description of the incidence of cancer associated with environmental exposure was published in 1775 by a London surgeon, Percival Pott. He attributed the unusually high incidence of scrotal cancer in young men, who were or had been chimney sweeps, to excessive exposure to soot during their work. Pott's hypothesis received further support when Butlin in 1892 observed a much lower incidence of scrotal cancer in chimney sweeps in the northern Europe compared to that of the English workers. Butlin concluded that the lower incidence of the cancer reflected the frequent bathing and the use of protective clothing among the northern European workers, which led to less exposure to soot. By the second half of the 19th century, development of skin cancer was reported in workers form many other occupations that involved body contact with coal tar and paraffin oils (Volkman, 1875; Bell, 1876; Manouvriez, 1876; as cited in Searle & Teale, 1990).

Aromatic amines started to be commercially produced following the synthesis of aniline and the aniline-based dyes in the middle 1800's. In 1895 German surgeon Rehn observed an increase in the incidence of urinary bladder cancer among the dyestuff workers, which he first thought to be

caused by exposure to aniline. Leichtenstern (1898), however, suggested that the bladder cancer might be associated with exposure to naphthylamines. Other reports linking the incidence of bladder cancer to dyestuff exposure started to appear as dyestuff factories expanded in other countries (Wignall, 1929; Gehrmann, 1934).

Clinical observations and epidemiological studies stimulated development of laboratory animal models to study the probable relationship between specific kinds of cancer and exposure to certain chemical compounds. The first direct proof that certain chemicals can provoke cancer by repetitive and excessive exposure was shown by Yamagiwa and Ichikawa in Japan, in 1915. They were able to induce skin carcinomas on the ears of rabbits by repeatedly applying coal tar to the rabbits' ears over a long period of exposure. Three years later, tar was also shown to be carcinogenic when applied to mouse skin, and in 1922, Passey showed that ether extracts of tars can also cause skin cancer in mouse (reviewed by Haddow & Kon, 1947).

The knowledge that tars and extracts actually can induce skin cancer, as well as Rehn's early observations on the "aniline" dye cancers indicating that relatively pure chemical compounds can be carcinogenic, stimulated the search for the active agent that causes the malignant transformation. In an attempt to identify the major carcinogenic component of coal-tar, Kennaway and Hieger (1930) were the first to synthesize and demonstrate the carcinogenicity of a synthetic polycyclic hydrocarbon, dibenzo[*a*,*h*]anthracene. Three years later, benzo[*a*]pyrene, a carcinogenic hydrocarbon, was isolated from coal-tar pitch (Cook et al., 1933). Benzo[*a*]pyrene, which results from the incomplete combustion of organic

materials, is now recognized as a widely distributed environmental carcinogen (National Academy of Science Reports, USA, 1972).

Aromatic amines were also extensively studied for their carcinogenicity in animals since Rehn's observation suggested "aniline" dyes were putative human carcinogens. In 1933, Yoshida succeeded in inducing liver tumors in rats and mice by feeding these animals with o-aminoazotoluene or 2',3-dimethyl-4-amino-azobenzene for extended periods of time. Soon after, an isomer, N-N-dimethyl-4-aminoazobenzene, was also shown to be a strong carcinogen (Kinosita, 1936). In 1938, Hueper and coworkers observed induction of bladder cancer in dogs fed 2-naphthylamine, confirming the suspicion of Leichtenstern about the carcinogenic nature of this compound.

By 1940 many aromatic amines and polycyclic hydrocarbons had been shown to be carcinogenic, either by experimental data from animal models, or by epidemiological studies in humans (Miller, 1978; Pitot, 1990). In 1940, 2-acetylaminofluorene (AAF) was patented as a potentially valuable insecticide, and it was submitted to toxicity studies in laboratory animals. One year later, Wilson et al. (1941) showed that AAF had little or no acute toxicity in rats, mice or rabbits. However, when albino rats were fed with AAF for 95 days or more, 50% of the rats developed malignant tumors in diverse organs, with a high incidence of bladder carcinoma. AAF was then banned from commercial use, but it has since been extensively studied as a model carcinogen for elucidating mechanisms of chemical carcinogenesis.

Since then, the list of known carcinogenic chemicals has expanded markedly, and new classes of chemicals have been shown to possess

carcinogenic activities. Alkylating agents (Lawley, 1976), dialkylnitrosamines (Magee et al., 1976), ethionines (Farber, 1963), pyrrolizine alkaloids (Cook et al., 1950, Shoental, 1976) and even inorganic chemicals (IARC, 1980; Gilman & Swierenga, 1984; Sunderman, 1984) have been proven to be carcinogenic. It also became clear that chemical carcinogens were not limited to products of technological advances of mankind such as high-temperature combustions or synthetic products from chemistry laboratories. Many naturally occurring substances, including certain metabolites of plants and microorganisms, have been shown to be carcinogenic in laboratory animals (Miller & Miller, 1976). We probably are constantly exposed to low doses of such substances through our food or from products of our intestinal flora.

The bigger and more diverse the list of chemical carcinogens became, the more difficult it became for oncologists to explain the mechanisms of cancer formation. Is there one mechanism by which all the carcinogens cause cancer or does each carcinogen have its unique mechanism? If the former were true, how was it possible since no common structural feature was observed?

One hypothesis that received wide acceptance among the scientists despite the controversies was the somatic mutation hypothesis. It states that the initiating event in the appearance of any cancer is a mutation within a somatic cell. The main obstacle to proving this hypothesis was the difficulty in obtaining conclusive data from experiments designed to study the carcinogenicity and mutagenicity of specific chemicals *in vivo*. Explanations for the difficulties experienced include the species differences of the models used, inappropriate routes of administration,

unsuitable solvents, and lack of appropriate parameters in the quantitative studies. Therefore, when Burdette reviewed the subject and evaluated the hypothesis in 1955, he indicated that among the many investigators who had tested a wide range of different compounds, no one had found any parallelism between mutagenicity and carcinogenicity. Therefore, he concluded that a general correlation between mutagenicity and carcinogenicity could not be proposed from the evidence that existed at that time.

However, a major breakthrough in the general understanding of the mechanisms of chemical carcinogenesis would come a few years later. In 1960, Cramer and the Millers observed the presence of a new metabolite in the urine of rats which were chronicaly fed AAF (Cramer et al., 1960; Miller et al., 1960). The new metabolite was identified as a glucuronide of *N*-hydroxy-2-acetylaminofluorene (N-OH-AAF), and when tested in rats, it was shown to be more carcinogenic than the parent compound, especially at sites of application (Miller et al., 1961; Miller et al., 1962; Goodal & Gasteyer, 1966). N-OH-AAF, however, did not react readily with tissue components at physiological pH (King et al., 1963; Miller et al., 1966), which was unexpected since the Millers had hypothesized that the binding of the carcinogen to cellular macromolecules constituted one the of first steps in the carcinogenic process (Miller & Miller, 1947; 1966). Interestingly, clarification of such controversial findings came from studies conducted with N-methyl-4-aminoazobenzene (MAB) which revealed that an ester derivative of MAB reacts readily with proteins and nucleic acids at pH 7 (Poirier et al., 1967). Meanwhile, synthetic neutral esters of N-OH-AAF were also shown to be highly reactive with nucleophilic sites

on proteins and nucleic acids (Miller et al., 1966). These studies led these investigators to propose the theory of step-wise metabolic activation of chemical carcinogens. According to their proposal, most chemical carcinogens could be considered "procarcinogens" which require metabolic activation to "proximate" carcinogens and finally to "ultimate" carcinogens, electrophilic reactants or radical cations that bind readily to cellular macromolecules, i.e., proteins and nucleic acids.

In 1968, Maher, working with Szybalski and the Millers was the first to demonstrate that well-known carcinogens, such as AAF and MAB, could in fact cause mutations if they were in the "ultimate" form (Maher et al., 1968). Their studies on the mutagenic activities of the esters of N-hydroxy-AAF and N-hydroxy-MAB indicated that mutations might participate, at least in part, in the mechanism of carcinogenesis by AAF and MAB. The general idea that no correlation existed between carcinogens and mutagens only reflected the lack of understanding of the need for metabolic activation.

A few years later, Ames and his collaborators developed a very sensitive and simple bacterial test for detecting chemical carcinogens which involved preparation and use of liver homogenates (S-9 fractions) as sources of carcinogen activation (Ames et al., 1973a). By 1975, more than 90% of the carcinogens tested had been shown to cause mutations in their assay, including aromatic amines, polycyclic hydrocarbons, direct alkalating agents, fungal toxins and other agents (Ames et al., 1975; McCann et al., 1975).

B. Critical characteristics of DNA adducts formed by reactive intermediates of 2-acetylaminofluorene

1. Kinds of adducts formed when reactive metabolites of AAF react with DNA

It is generally accepted that the active form of a carcinogenic agent must interact in some manner with crucial cellular components in the induction of cancer (Miller & Miller, 1947; 1966; 1969a; 1969b). Since nucleic acids play a central role in storing, replicating and transferring the genetic information, studies on their interaction with carcinogens are of major importance to an understanding of the molecular mechanisms of carcinogenesis (Faber, 1968; Miller & Miller, 1969a; 1969b). Incorporation of ^{14}C into hepatic DNA and RNA was demonstrated when rats were administered ^{14}C radiolabeled AAF or its more carcinogenic metabolite, N-OH-AAF (Williard & Irving, 1964; Sporn & Dingman, 1966; Kriek, 1968). Since neither AAF nor N-OH-AAF reacted *in vitro* with nucleic acids at physiological pHs (Miller et al., 1966), some further metabolic activation seemed to be required for the reaction. *N*-hydroxy-2-aminofluorene (N-OH-AF), a *in vivo* metabolite of AAF (Kriek, 1965) and N-OH-AAF (Irving, 1966) were shown to react with guanine nucleotides of RNA and DNA *in vitro* at pH 5-6. Meanwhile, another *in vivo* metabolite of N-OH-AAF, *N*-acetoxy-2-acetylaminofluorene (N-AcO-AAF) was shown to react readily with guanine and guanosine *in vitro* at pH 7 (Miller et al., 1966). The product of guanosine and N-AcO-AAF interaction has been identified as *N*-(guanosin-8-yl)-2-acetylaminofluorene (Kriek et al., 1967), which is analogous to the structure proposed by Kriek (1965) for the reaction of guanine derivatives

with N-OH-AF. A similar reaction was observed between N-AcO-AAF and guanine in soluble ribonucleic acid (sRNA) and deoxyribonucleic acid (DNA) (Kriek et al., 1967). *N*-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF) was the only adduct detected when products of the DNA interaction with [G-^{3}H]N-OH-AF were hydrolyzed in dry trifluoroacetic acid (TFA) and analyzed by high-pressure liquid chromatography (HPLC) (Westra & Visser, 1979). Using this same procedure to analyze the products of the interaction between DNA and [G-^{3}H]N-AcO-AAF, 70% of the total DNA-bound radioactivity was recovered as *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-C8-AAF) (Westra & Visser, 1979) and the remaining 30% of the radioactivity consisted of the isomeric N^2-substituted guanine compound (Westra et al., 1976; Westra & Visser, 1979).

When 2-aminofluorene (AF), AAF, or their *N*-hydroxy derivatives were administered to either laboratory animals or mammalian cells in culture, three DNA adducts were formed (Irving & Veazey, 1969; Kriek, 1969; 1972; Howard et al., 1981; Poirier et al., 1982). The major DNA adduct formed in rat liver *in vivo* (80%), and the only adduct formed in the rat mammary gland was a nonacetylated product dG-C8-AF (Irving & Veazey, 1969; Kriek, 1969; 1972; Allaben et al., 1983). Formation of dG-C8-AF is thought to result from the metabolic production of *N*-acetoxy-2-aminofluorene (N-AcO-AF) through a cytosolic or microsomal *N,O*-acyltransferase-catalyzed reaction (Bartsch et al., 1972; King,1974; Beland et al., 1980; Shirai et al., 1981; Allaben et al., 1983; Wang et al., 1988). Theoretically, dG-C8-AF could also arise from deacylation of N-OH-AAF to give the reactive derivative N-OH-AF (Schut et al., 1978; Frederick et al., 1982). However, experiments involving the use of a deacylase inhibitor, paraoxon, did not

decrease the level of dG-C8-AF when rat hepatocytes were incubated with N-OH-AAF (Howard et al., 1981). On the other hand, mice have very low *N,O*-acyltransferase activity (King & Glowinski, 1983), and recent studies have shown that in this species, the N-OH-AAF is deacetylated to N-OH-AF, which is then converted into a reactive *N*-sulfonyloxy ester (Lai et al., 1985; 1987).

The two *N*-acetylated adducts formed *in vivo*, dG-C8-AAF (15%) and 3-(deoxyguanosin-N^2-yl)-2-acetylaminofluorene (dG-N^2-AAF) (5%) are generated from a reactive sulfate intermediate in rat liver (DeBaun, 1970; Westra et al., 1976; Shirai & King, 1982). Data from experiments with the sulfotransferase inhibitor pentachlorophenol confirm this fact (Meerman et al., 1981; Lai et al., 1985; 1987). Another possible pathway for formation of the acetylated adducts is through an *O*-acetylated intermediate, N-AcO-AAF, which is formed by the direct reaction of acetyl coenzyme A with N-OH-AAF (Lotlikar & Luha, 1971; Flammang & Kadlubar, 1983). However, when mammalian cells in culture are treated with N-AcO-AAF, the dG-C8-AF adduct is the principal adduct formed (Maher et al., 1980; Poirier et al., 1980; Carothers et al., 1986; 1989a; Heflich et al., 1988), probably as a result of the *N*-deacetylation of N-AcO-AAF to N-AcO-AF (Heflich et al., 1988).

A number of minor adducts have also been reported both *in vitro* and *in vivo*, including an adenine adduct of unknown structure which has been observed in *in vitro* studies using polyadenylate or DNA. However, none of these adducts has been characterized extensively (Kapuler & Michelson, 1971; Kriek & Reitsema, 1971; Harvan et al., 1977; Gupta & Dighe, 1984).

2. Physico-chemical effects of AF and AAF adducts on the DNA structure

Since at least 3 kinds of adducts are formed upon the interaction of reactive metabolites of AAF with DNA, studies were carried out to investigate the mechanisms by which these adducts might distort nucleic acid structure and consequently affect biological activity. The presence of dG-C8-AAF adducts in the G-U-U and A-A-G codons, as well as in poly (UG) was shown to inhibit the ability of these oligomers to stimulate ribosomal binding of their respective aminoacyl tRNA's (Grunberger et al., 1970; Grunberger & Weinstein, 1971). Circular dichroism data suggested that AAF binding to nucleic acids involved important conformational changes that would affect the function of DNA or RNA (Grunberger et al., 1970). Further studies on the conformational properties of various oligonucleotides modified with AAF were carried out using circular dichroism, proton magnetic resonance spectroscopy, and computer generated molecular models. These studies showed that covalent binding of AAF to the C-8 position of guanine caused two major changes in the conformational properties of oligonucleotides in aqueous solution. The guanine residue bearing AAF-adduct was rotated around the glycosidic linkage, assuming the *syn* conformation instead of the normal *anti* conformation, and stacking interactions between the AAF and the base adjacent to the guanine was observed (Nelson et al., 1971). The "base displacement" model was proposed to explain these conformational changes (Levine et al., 1974).

AAF adduct-induced DNA structural alterations were also found by Fuchs and his group. Local denaturation of DNA induced by the adduct was detected by decreases in melting temperature (Fuchs & Daune, 1972), and by changes in formaldehyde unwinding kinetics (Fuchs & Daune, 1974).

Fuchs (1975) proposed an "insertion-denaturation" model where the fluorene ring was accommodated between the two nearest "base plates", and Fuchs & Daune (1974) showed that the extent of denaturation included about 12 base pair.

In contrast to dG-C8-AAF residues , a great variety of conformations are available for dG-C8-AF residues. Several structures have been proposed, including an outside binding model as well as an insertion model similar to that described for dG-C8-AAF (Spodheim-Maurizot et al., 1979; 1980; Evans et al., 1980; Broyde & Hingerty, 1983). Minimized potential-energy calculations showed that the AF adduct on C-8 of dCpdG was much more flexible than the AAF adduct, with many low-energy conformers in both *anti* and *syn* domains of the guanine glycosidic rotation (Broyde & Hingerty, 1983). Any or all of these conformations may exist, but the most important feature is that AF residues bound to the C-8 position of guanine induce less distortion in conformation of the modified regions than do AAF residues (Santella et al., 1980; Melchior & Beland, 1984).

Although dG-N^2-AAF residues have not been studied by the techniques cited above, Grunberger & Santella (1981) found that the N^2 adduct is much less susceptible to S_1 nuclease digestion than the dG-C8-AAF, suggesting that dG-N^2-AAF causes much less distortion of the DNA helix. Also a theoretical study has suggested that the AAF adduct at the N^2 position of guanine can reside without steric hindrance in the minor groove of the DNA double helix (Beland, 1978).

Another relevant feature of an AAF residue bound to the C-8 position of guanine is its ability to induce formation of Z-DNA which implies certain biological consequences that will be discussed in the next

section. When AAF binds to synthetic polynucleotides with alternating pyrimidine-purine sequences (such as CG or TG), it triggers the conversion of the polynucleotide from the B-form normally found in DNA to the Z-form (Sage & Leng, 1980; 1981; Santella et al., 1981a; 1981b; Wells et al., 1982). This confromational change, presummed to decrease steric interactions, probably results from the tendency of dG-C8-AAF adducts to induce the rotation of the guanine residue to the *syn* conformation. Theoretical calculations suport this hypothesis (Hingerty & Broyde, 1982). B --> Z transitions have also been observed in dG-C8-AF-modified polynucleotides, however, considerably higher salt or alcohol concentrations were required (Sage & Leng, 1980).

3. Biological properties of AF and AAF adducts

Since the demonstration by Wilson et al. (1941) of the tumor induction ability of AAF, this compound and its metabolic derivatives have been extensively used in animal carcinogenesis studies (Weisburger & Weisburger, 1958; Miller, 1978). N-AcO-AAF also has been shown to induce homologous recombination in bacteria (Luisi-DeLuca et al., 1984) and mammalian cells (Bhattachryya et al., 1989).

The mutagenic action of N-AcO-AAF, N-OH-AAF or N-OH-AF in bacteria (Ames et al., 1972b; Beranek et al., 1982), human cells (Maher & Wessel, 1975; Aust et al., 1984) and other mammalian cells (Carothers et al, 1986; 1989a; Heflich et al., 1988) is well documented and will be discussed in more detail in section C. However, it should be pointed out here that dG-C8-AAF was shown to cause mainly frameshift mutations in the tetracycline resistance gene of *E. coli* inserted into the pBR322 plasmid (Koffel-

Schwartz et al., 1984), whereas dG-C8-AF induced primarily base substitution mutations in the same gene (Bichara & Fuchs, 1985). It was proposed by these investigators that the major conformational alterations triggered by dG-C8-AAF by either the "insertion-denaturation" model or the induction of Z-DNA would favor the induction of frameshift mutations, while the minor alterations induced by dG-C8-AF would result primarily in base substitutions (Koffel-Schwartz et al., 1984; Bichara & Fuchs, 1985). Recent studies by Freund et al (1989) showed that plasmids containing sequences capable of adopting the Z conformation (GC and CA/GT) were hot spots for spontaneous deletions.

In vitro DNA polymerization studies by Strauss and coworkers involving single stranded bacteriophage DNA containig dG-C8-AF and dG-C8-AAF indicated that these adducts differ in their ability to interfere with polymerization. The dG-C8-AAF caused the T4 DNA polymerase to terminate DNA synthesis one nucleotide before the adduct, whereas, the deacetylated adduct induced polymerase termination after incorporation of the nucleotide opposite to the modified guanine (Moore & Strauss, 1979; Moore et al., 1982). This difference was explained by the known preferred conformations assumed by the two adducts. A similar differentiation between the two adducts was also found with *E. coli* DNA polymerase I (Klenow fragment), and with the modified T7 DNA polymerase (Sequenase). The latter polymerase terminates DNA synthesis one nucleotide before dG-C8-AAF, however, it seems sometimes to be able to bypass dG-C8-AF lesions (Sahm et al., 1989). AAF adducts were also shown to severely block the progression of the replication forks in Simian Virus 40 (SV40) DNA by inhibiting the *in vivo* polymerization of the leading strand (Armier et

al., 1988).

The differences in the chemical characteristics of AF and AAF adducts also seem to affect the way these adducts are handled by cellular DNA repair systems. Although each of these adducts is chemically stable (Johnson et al., 1987), they are removed from the DNA by DNA excision repair enzymes at different rates. The acetylated C-8 adduct is rapidly removed from rat liver *in vivo*, with a half-life of approximately 7 days (Kriek, 1972). This was also the case in primary cultures of rat hepatocytes where the dG-C8-AAF adducts were removed with a half-life of approximately 10 hr (Howard et al., 1981). In contrast, the dG-N^2-AAF and dG-C8-AF are relatively persistent adducts in this animal. The N^2-deoxyguanosine adduct is maintained at a constant level for at least 8 weeks in rat liver *in vivo* (Kriek, 1972). In primary rat hepatocyte cultures this residue remains at a steady concentration for at least 14hr and then is slowly removed, with a half-life >40 hr. In this same experiment, the rate of removal of dG-C8-AF was very similar to that of dG-N^2-AAF (Howard et al., 1981). Although dG-C8-AF adducts were not detectable after four weeks following a single injection of AAF in Kriek's experiment (Kriek, 1972), Beland and coworkers have found a major product that comigrates with dG-C8-AF two weeks after multiple doses of N-OH-AAF (Beland et al., 1979). Furthermore, the frequency of dG-C8-AF adducts was shown to remain essentially constant after Wistar-Furth rats had been fed AAF for 28 days (Poirier et al., 1982). The difference in the persistence of the adducts is probably associated with the major DNA conformational alterations caused by dG-C8-AAF adducts which would serve as a signal for the DNA excision repair enzyme system that removes the adduct (Grunberger

& Santella, 1981; Beland & Kadlubar, 1990). Boiteux et al. (1985) found that the conversion of a polynucleotide containing O^6-methylguanine to the Z-form completely inhibited the repair of the adduct, implying that B --> Z transitions may decrease the rate of adduct repair, and therefore, the original structural distortions in the B-DNA bearing dG-C8-AAF is probably the important determinant in adduct removal. Furthermore, the excision of dG-C8-AAF adduct in *Escherichia coli* was shown to require all three genes of the error-free DNA excision repair *uvrABC* system, whereas this was not the case with dG-C8-AF (Tang et al., 1982).

The carcinogenic potency of a certain chemical compound seems to depend not only on its ability to bind to DNA and alter its template function, but also on its capacity to bind in a certain way that does not induce the error-free cell DNA repair systems. However, there is no simple correlation between the persistence of DNA adducts *per se* and induction of tumors, since persistence of dG-C8-AF was observed in rat kidney and female rat liver which are tissues resistant to *N*-OH-AAF-induced tumorigenesis (Beland et al., 1982).

C. Experimental systems that have been used to investigate mechanisms of AAF mutagenesis

It is now generally recognized that the transformation of normal cells into tumorigenic cells is a multistep process, and there is substantial evidence that mutations are involved in causing one or more of the changes required for carcinogenesis (Zarbl et al., 1985; Baker et al., 1989; Horowitz et al., 1989). However, the molecular mechanisms by which carcinogens induce mutations in mammalian cells are not well

understood. Due to the simple organization of prokaryotic cells, extensive genetic studies have been done using prokaryotic genes. One of these is the *E. coli lacI* gene which has been extensively characterized, and has been widely used to investigate both spontaneous and induced mutations in bacteria (Calos & Miller, 1981; Miller, 1982). The well-developed techniques of bacterial molecular genetics, combined with DNA sequence analysis, provide a simple way of obtaining precise mutational information on prokaryotic systems. On the other hand, the inherent complexity of higher eukaryotic cells make it very difficult to obtain information at the DNA sequence level on mutations in mammalian cells. The development of recombinant DNA techniques during the last decade have enabled researchers to generate vectors exogenous to the target cell which make it possible to study mutational changes in eukaryotic genes by direct nucleic acid analysis. These vectors have to rely totally on the host cell DNA repair and DNA replication machinery, and therefore can be used to investigate the host cell's mutagenesis activity based on the assumption that mutations are produced when DNA synthesis occurs on a damaged DNA template (Konze-Thomas et al., 1982; Grossman et al., 1985). The development of shuttle vectors, plasmids that can replicate in both eukaryotic and prokaryotic cells, have provided the tools for studying mutagenesis in mammalian cells (Calos et al., 1983; Razzaque et al., 1983; 1984; Lebkowski et al., 1984; Seidman et al., 1985). These plasmids carrying target DNA sequences can be exposed to mutagens or carcinogens, and introduced into eukaryotic cells, and then the progeny plasmids can be rescued and assayed for mutants in bacteria and the mutations analyzed by DNA sequencing. Recent development of the polymerase chain reaction

technique (Carothers et al., 1989b; Yang et al., 1989), which enables one to amplify specific endogenous genes, offers a still more elegant means of studying specific mutations in eukaryotic cells.

1. Plasmid and viral vector systems in bacterial cells

A general strategy for studying mutagenic mechanisms is to determine the mutational specificity, i.e., the precise nature and locations of mutations produced by the carcinogen. The simplest way to study mutational specificity consists of determining the forward mutation spectrum in a specific gene after it is randomly modified with a mutagen producing a readily identifiable phenotype (Fuchs et al., 1988). This approach was used by Fuchs and coworkers to study the specific mutations induced by AF and AAF adducts when plasmids carrying such adducts replicated in the bacterium *E. coli*. Plasmid DNA was treated in vitro with derivatives of AAF which formed almost exclusively AF or AAF adducts. The assay used the plasmid pBR322 containing the tetracycline-resistance gene as a target for mutagenesis. The mutagen-treated plasmids were introduced into *E. coli* by transformation, and tetracycline-sensitive transformants were selected and sequenced. Their results showed that more than 90% of the mutations induced by AAF adducts were frameshifts and were located within specific sequences, so called "hot spots" (Fuchs et al., 1981; Koffel-Schwartz et al., 1984). In contrast, 85% of mutations induced by AF adducts were base substitutions, mainly G C --> T A transversions (Bichara & Fuchs, 1985). Comparison of the AAF binding spectrum to the AAF mutational spectrum showed no direct correlation between DNA modification and the mutation event, suggesting that the mutational "hot spots" may have resulted from

the processing of an unusual DNA conformation induced by the premutagenic lesion within specific DNA sequences. Such "hot spot" sequences were either runs of guanines in which the mutations were mainly minus one base pair deletions or the sequence GGCGCC, which is recognized by the restriction enzyme *Nar*I, and in which the mutations consisted of minus two frameshifts (Koffel-Schwartz et al., 1984).

To further study the mechanisms of AAF-induced mutations at NarI sites, oligonucleotides containing a single adduct at each of the three guanines at the *Nar*I site were ligated into a gapped-duplex pSM14 plasmid, resulting in plasmids carrying a unique AAF adduct at each of the three positions of the *Nar*I site (Koehl et al., 1989). Using these plasmids they found that only the AAF adduct formed at the third guanine residue in the 5′ to 3′ direction induced the minus two base pair deletion. They also concluded that the *Nar*I mutational "hot spots" belong to a family of sequences that are processed by a specific mutation pathway which is different from the pathway for base substitutions and from the Streisinger-type of frameshift which occurs at repetitive sequences (Burnouf et al., 1989). Further studies using DNA fragments containing single AAF modified guanines within the *Nar*I site showed that the efficiency of excision repair of AAF adducts depends on the context of the DNA sequence, and ineffectively repaired adducts do not necessarily represent mutational hot spots (Seeberg & Fuchs, 1990).

M13 bacteriophages and pBR322 plasmids have been used by many other investigators to study the mechanisms of AAF mutagenesis. In some studies the plasmid or viral vector was treated with N-OH-AF or N-AcO-AAF, resulting in DNA containing AF or AAF adducts randomly distributed along

the whole vector. In other studies, the bulky adduct dG-C8-AF or dG-C8-AAF was introduced into defined sites in the target gene, resulting in a vector which carries a site-specific adduct. Gupta et al. (1988) used single-stranded DNA of the bacteriophage M13mp8 and its complementary double-stranded DNA, randomly modified with AF adducts in a forward mutation assay to study the mutational specificity of N-OH-AF in an *E. coli* system. DNA sequence analysis of the mutants showed that 52-55% of the mutations induced in this assay were base substitutions, which is similar to Bichara & Fuchs' (1985) data when pBR322 containing random AF adducts was allowed to replicate in *E. coli*. However, a significant number of large deletions (25-30%) and frameshifts (16-18%) were also reported by Gupta et al. (1988).

Some investigators have used classical recombinant DNA techniques to clone site-specific modified double-stranded duplexes into plasmid DNA. Mitchell & Stohrer (1986) synthesized a 17 base pair long double-duplex DNA containing a site-specific dG-C8-AF which was ligated with the large *ECO*RI-*Bam*HI fragment of plasmid pBR322. The site-specifically modified oligonucleotide consisted of the shortest active form of the *E. coli* lactose operator and served as the target for mutagenesis. Mutations in the cloned operator abolish its function, restoring the inducible, but repressed phenotype of the *E. coli* lactose operon. Ten randomly selected mutants were analyzed by DNA sequence, of which nine showed a single base pair deletion and one showed a two base pair deletion. Moriya et al. (1988) also made use of classical recombinant DNA techniques to clone into a shuttle vector the tetracycline-resistance gene carrying a site-specific dG-C8-AAF. This shuttle vector was then used to transfect simian kidney

kidney cells and to transform several AB strains of *E. coli*. DNA sequence analysis of the mutants obtained after shuttle vector replication in *E. coli* indicated that 85-90% of these contained alterations at the site of dG-C8-AAF, and 70-80% of such changes were base substitutions, predominantly G·C --> C·G transversions. Johnson et al. (1986; 1987) used gapped heteroduplex precursors for placing site-specific dG-C8-AF or dG-C8-AAF lesions into M13 bacteriophage genomes. When these viral vectors were assayed for mutations after replication in *E. coli*, both site-specific adducts were found to be capable of inducing base substitutions at the site of modification. However, a specific plus one base pair insertion was also observed at the ligation site (Gupta et al., 1989). The results of these studies using site-specific adducts suggest that mutagenesis depends not only on the structure of the adduct, but also the sequence in which the adduct is located and the host cell type used for mutation expression.

2.Endogenous gene systems in bacterial cells

The mutagenic potency of AAF was first shown by Maher et al. in 1968. By treating biologically active DNA isolated from *Bacillus subtilis* with a series of metabolites and synthetic derivatives of the carcinogen AAF, they showed severe reductions in transforming ability of the treated DNA proportional to the doses applied. Increases in the frequencies of mutations as a function of the inactivation of the transforming DNA by derivatives of AAF were observed as well. These data indicated that the apparent lack of correlation between carcinogens and mutagens that was previously observed simply reflected the necessity of metabolic

sensitive and simple bacterial test for detecting chemical carcinogens (Ames et al., 1973a). It was clear that the principal limitation of bacterial systems in detecting carcinogens as mutagens was the inability of the bacterial cells to metabolically activate the carcinogens. Some researchers have made use of mammalian liver homogenates to activate chemical compounds of interest (Malling, 1971; Slater et al., 1971; Garner et al., 1972).

Ames and his group extended the studies and showed that the simple incubation of a rat or human liver homogenate with a carcinogen and a bacterial tester strain in a petri dish can detect mutagens with great sensitivity (Ames et al., 1973b). By using this liver homogenate activating system, the mutagenic potency of a series of metabolic derivatives of 2-AAF was investigated. Four histidine-dependent strains of *Salmonella typhimurium* were used in a reversion mutation assay. Three of the strains were designed to detect frameshift mutations with different specificities since each of them carries a frameshift mutation in one of the genes of the histidine operon. The fourth strain was used to detect base substitution mutations. The results showed that no mutations were scored with the base substitution tester strain, while some of the metabolites of AAF, including N-OH-AAF, N-OH-AF and N-AcO-AAF were strong frameshifters. From this data and previous findings (Ames et al., 1972a; 1972b), they conclude that these derivatives of AAF belong to a particular class of potent frameshift mutagens that not only intercalates in the DNA base pair stack, but can also react covalently with the DNA, enhancing the mutagenic potency. These compounds apparently induce frameshift mutations by stabilizing a shifted pairing in the DNA duplex

frameshift mutations by stabilizing a shifted pairing in the DNA duplex (Ames et al., 1972b).

Beranek et al. (1982) used *S. typhimurium* strain TA 1538 to investigate the kinds of DNA adducts formed when these bacteria were incubated with the carcinogen N-OH-AAF in the presence and absence of rat liver homogenate, as well as the biological significance of the adducts in a mutation assay. The strain TA 1538 can detect frameshift mutagens and it has a repetitive CGCGCGCG sequence near the site of the histidine mutation (Ames et al., 1975). A correlation between mutagenicity and the extent of dG-C8-AF adduct formation was observed. Since this was the only adduct detected in their experiment, and it was also the major adduct found in rat liver in vivo after administration of N-OH-AAF (Irving & Veazey, 1969; Kriek, 1969; 1972), they concluded that dG-C8-AF adducts might be a critical lesion for the initiation of hepatic tumorigenesis (Beranek et al., 1982).

More recently, Fuchs and his group, after extensive studies of the mutagenesis of AF and AAF adducts in the tetracycline-resistance gene of plasmid pBR322, as well as the specific AAF-induced mutations at *Nar*I sites, extended the studies of AAF mutagenesis to a endogenous bacterial gene. They chose the *E. coli lacI* gene which encodes the repressor of the lactose operon. *lac*$^-$ mutants are readily identifiable because of their constitutive expression of the operon. This gene is about 1100 base pair long and is a well-studied mutational target in which point mutations as well as larger mutations are readily scored. Since the *lacI* gene is about 4 times larger than the previously used tetracycline-resistance gene and also differs from the former one in base composition, it should be a

useful tool in understanding the general characteristics of AAF mutagenesis. An *E. coli* strain permeable to the ultimate carcinogen N-AcO-AAF was treated with the carcinogen which offers the *in vivo* context for the reaction of carcinogen with DNA. However, in this circumstance, the spectrum of DNA adducts cannot be controlled as well as in *in vitro* approaches. They previously showed that when bacteria were treated with the ultimate carcinogen N-AcO-AAF, 40% of the adducts were dG-C8-AAF, whereas the remaining were in the deacetylated form (Salles et al., 1983). This study of AAF-induced mutagenesis revealed several key features that were previously observed in the pBR322 system. Within the point mutations, base substitutions and single base deletions were observed. But most importantly, 50% of the point mutations represented minus two frameshifts tightly linked to alternating purine-pyrimidine sequences, predominantly of the $(GpC)_n$ type, which strengthened the importance of frameshift mutagenesis at alternating purine-pyrimidine sequences. Only 44% of the mutations scored in the *lacI* system were point mutations, which could be partially a reflection of the increase in the size of the target gene. However, N-AcO-AAF has been shown to be able to induce large deletions in *Drosophila* (Fahmy & Fahmy, 1972) and in cultured hamster cells (Carothers et al., 1986), suggesting that larger mutations may be of general importance in the AAF induced mutagenesis (Schaaper et al., 1990).

3. Shuttle vector and viral vector systems in mammalian cells

Despite the simplicity and convenience that exogenous probes such as shuttle vectors and viral vectors offer for mammalian cell mutagenesis studies, only a few researchers have taken advantages of these systems to

Gentil et al. (1986) used a reversion assay involving a temperature-sensitive SV40 tsA58 mutant to determine the mutational specificity of AAF on the SV40 large tumor (T) antigen gene. The SV40 virions were treated *in vitro* with N-AcO-AAF and allowed to infect monkey kidney CVP-1 cells. The mutation assay was based upon the reversion of the SV40 tsA58 mutants from a temperature-sensitive growth phenotype to a wild-type growth phenotype at 41°C. N-AcO-AAF treatment induced both a decrease in progeny survival and an increase in viral mutagenesis. Molecular analysis of independent SV40 revertants showed that AAF induced base substitutions which were not located opposite AAF adducts, but next to them, and more than 60% of the mutants were located at one hot spot, which was also not opposite a major DNA lesion. To explain this sequence-specific "hot spot" they proposed two models which involved the stabilization of the secondary structure of a specific quasipalindromic SV40 sequence by AAF adduct.

As mentioned above, Moriya et al. (1988) used classical recombinant DNA techniques to clone site-specific AAF modified double-stranded duplexes into the tetracycline-resistance gene of a shuttle vector to study AAF induced mutagenesis in mammalian cells. Shuttle vectors carrying the dG-C8-AAF adducts were used to transfect SV40-transformed simian kidney COS-1 cells. The presence of a single AAF adduct increased the mutation frequency by 8-fold and more than 80% of the mutations were targeted to the adduct. The results showed that 90% of the alterations induced by AAF adducts in COS cells were base substitutions, and 95% of these represented either G•C --> T•A or G•C --> C•G transversions, changes that occurred at similar frequencies.

4.Endogenous mammalian gene systems

Carothers et al. (1986) have studied the types of alterations in gene structure induced by the carcinogen N-AcO-AAF in the endogenous dihydrofolate reductase (*dhfr*) gene of cultured Chinese hamster ovary cells. A profile of the 26-kilobase long gene was obtained by Southern blot analysis of the mutant DNA. From twenty-nine independent enzyme-deficient mutants isolated, twenty-one (72%) contained small mutations (changes <100 base pairs in size). The remaining 28% of the mutants presented deletions and complex gene rearrangements. HPLC analysis of enzymatically hydrolyzed DNA from cells incubated with tritiated N-AcO-AAF under the same conditions used for mutagenesis showed that 88% of the adducts were dG-C8-AF and 12% were dG-C8-AAF. They proposed that a correlation existed between the conformational alterations induced in DNA by the two adducts and the type of mutations identified at the *dhfr* gene. DNA from eleven of the mutants were later amplified by the polymerase chain reaction technique and sequenced. DNA sequence analysis showed that 90% of these mutants carried base substitutions, of which 75% were G•C --> T•A transversions. The remaining sequenced mutants contained frameshift mutations (Carothers et al., 1989a).

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CHAPTER II

Mutations Induced by Aminofluorene-DNA Adducts During Replication in Human Cells

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SUMMARY

To gain insight into the mechanisms by which carcinogens induce mutations in human cells, we treated a shuttle vector, pZ189, carrying the *supF* gene as the target for mutations with *N*-trifluoroacetyl-2-aminofluorene (N-AcO-TFA-AF). The plasmids were allowed to replicate in human cell line 293, and the progeny plasmids were examined for the frequency and kinds of mutations induced in *supF*, as well as their specific location in the sequence of the *supF* gene. The plasmids were reacted with *N*-AcO-TFA-AF so as to obtain the deacetylated adduct *N*-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF), the principal adduct formed in DNA when mammalian cells are exposed to reactive derivatives of 2-acetylaminofluorene (AAF), including *N*-acetoxy-2-acetylaminofluorene. The results showed there was a linear relationship between the number of dG-C8-AF adducts per plasmid and the frequency of *supF* mutants induced. DNA sequencing of 47 independent mutants obtained from doses of N-AcO-TFA-AF that increased the frequency of mutants 9 to 15 times the background frequency and 3 independent mutants from lower doses showed that 92% contained point mutations, i.e., changes affecting one, or two, or three nearby bases, and that all of these point mutations involved G•C base pairs. 98% of the point mutations were base substitutions, predominantly G•C → T•A transversions. 46% of these mutations occurred at 4 out of the 85 base pairs in the target gene (hot spots). The most prominent mutation hot spot was also the most prominent hot spot for adduct formation as judged by the frequency of termination of *in vitro* polymerization by the Klenow fragment on *N*-AcO-TFA-AF-treated plasmids.

INTRODUCTION

It is now widely recognized that the transformation of normal cells into tumorigenic cells is a multistep process, and there is substantial evidence that mutations are involved in causing one or more of the changes required for carcinogenesis (1-3). However, the molecular mechanisms by which carcinogens induce mutations in mammalian cells are not well understood. Attempts to decipher the mechanisms involved have been hampered in the past by an inability to isolate newly mutated genes and analyze the changes at the sequence level. But the development of shuttle vectors, plasmids that can replicate in mammalian cells and also in bacteria (4-6), as well as the recent development of methods for amplifying specific endogenous genes using the polymerase chain reaction (7,8), have overcome this problem.

To examine the frequency and kinds of mutations induced by a series of structurally-related carcinogens, as well as their specific location in the target gene (spectrum of mutations), Maher and her colleagues (9-11) have been making use of a shuttle vector, pZ189, containing a target gene, *supF* , which is treated with radiolabeled carcinogens and then allowed to replicate in the human kidney cell line 293 where the mutations are introduced. The purpose of such studies is to compare the spectra of mutations induced in order to identify common features in the modes of action of the various agents and determine how they differ from each other. The use of the *supF* gene, which codes for a tRNA, as the target gene for these mutation studies offers several advantages. The structure of the tRNA is essential for its purpose, and extensive studies with the *supF* tRNA gene have demonstrated that a single base pair (bp) change at

almost any site in the 85 bp structure of the tRNA results in a mutant phenotype (reviewed in ref. 12), making this gene exceptionally responsive to mutagens and allowing for very few silent mutations. For example, there are 50 G•C bp in the structural gene, and a base pair substitution at any one of 43 of these has been shown to give a phenotypic change (12). Five others have been found to give a phenotypic change when a second base pair substitution was also present (12). The small size of the target gene greatly facilitates sequence analysis and determination of "hot spots" and "cold spots" for mutation induction by particular carcinogens.

Maher and her colleagues have studied (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) (9) which binds principally to guanine at the N2 position (13), and 1-nitrosopyrene (1-NOP) (10) which also reacts almost exclusively with guanine, but at the C8 position (14,15). Bigger et al. (16) used a closely related plasmid, pS189, and the human 293 cell line to obtain the spectrum of mutations induced in the *supF* gene by unlabeled benzo(c) phenantherene (4R,3S)-dihydrodiol (2S,1R)-epoxide [(-)-BcPhDE-2] which binds to adenine and guanine. In the present study, we have investigated the mutational spectrum obtained with N-(deoxyguanosine-8-yl)-2-aminofluorene (dG-C8-AF), the predominant adduct found when mammalian cells are exposed to reactive derivatives of 2-acetoxylaminofluorene, including N-acetoxy-2-acetylaminofluorene (N-AcO-AAF) (17-21). To obtain this deacetylated adduct, rather than the acetylated adduct dG-C8-AAF, we reacted the shuttle vector with rediolabeled N-acetoxy-N trifluoroacetyl-2-aminofluorene (N-AcO-TFA-AF). We have obtained a linear relationship between the number of AF adducts per plasmid and the frequency of *supF* mutants induced. All of the

mutations involved G·C bp, and 98% were base substitutions, predominantly G·C → T·A transversions. The frequency of mutants induced per C8 guanine AF adduct equaled that observed previously by Yang et al. (10) with 1-NOP.

MATERIALS AND METHODS

Cells and Plasmid. The human embryonic kidney cell line, 293, which served as the eukaroytic host for the shuttle vector, as well as the ampicillin sensitive indicator bacteria host (SY204) carrying an amber mutation in the chromosomal -galactosidase gene, have been described (9). The 5504-bp shuttle vector used, pZ189, constructed by Seidman et al. (5) contains the tyrosine amber suppressor tRNA structural gene (*supF*) flanked by two genes essential for its recovery in bacteria under the selective conditions, i.e., the gene for ampicillin resistance and the bacterial origin of replication. pZ189 also contains the origin of replication and large tumor antigen gene from simian virus SV40 which facilitates its replication in the 293 cells.

Formation of AF Adducts on the Plasmid. Plasmids were prepared by using an alkaline lysis procedure (22) and purified by ethidium bromide - CsCl density gradient centrifugation. DNA dissolved in 2 mM sodium citrate buffer, pH 7.0, at 1 mg/ml was added to a freshly prepared ethanol solution of generally-tritiated N-AcO-TFA-AF (60 mCi/mmole) (23) and incubated at 37°C for 30 min. The unbound compound was removed by several successive ether extractions, followed by extensive purification using phenol buffered (pH 7.0) with sodium citrate and finally by ethanol precipitation. The DNA was dissolved in sodium citrate buffer pH 7.0 and stored at -20°C until used. The number of residues bound per mole of plasmid was calculated from the A_{260} absorption profile of the DNA and the specific activity of the carcinogen, using 1 OD = 50 ug DNA/ml.

Determination of Kinds of Adducts Formed. The adducts were characterized following hydrolysis with trifluoroacetic acid as described (18). An aliquot of carcinogen-treated pZ189 was dried under vacuum. One ml of anhydrous trifluoroacetic acid was added and the DNA was incubated at 70°C for 1 hr. The trifluoroacetic acid was evaporated, and 150 ul of 80% methanol was added. Recovery was ~ 80%. The products of the hydrolysis were analyzed by reverse-phase HPLC on a Waters C_{18} uBondapak column using a 0-100% methanol/water gradient in 30 min at a flow rate of 1.0 ml/min. Fractions to be analyzed by scintillation counting were collected every 30 sec.

Transfection and Rescue of Replicated Plasmid. The procedures used to introduce the plasmids into the 293 cells and rescue the progeny plasmids were essentially as described (9). Briefly, the cells were plated into a series of 150-mm diameter dishes at a density of 2 - 3 x 10^6 cells/dish, incubated under growth conditions for 24 hr, and transfected with 6 g of treated or untreated plasmid per dish using calcium phosphate co-precipitation. The cells were allowed to grow for an additional 48 h before low molecular weight DNA (plasmid) was extracted and purified. Plasmids harvested from individual dishes were kept separate from each other so that we could distinguish between mutations that occurred frequently (hot spots) and putative siblings arising from within the same population. Prior to bacterial transformation, the plasmids were treated with DpnI to digest any DNA that still had the methylation pattern of the bacteria used to prepare the original input plasmid.

Bacterial Transformation and Mutant Characterization. The techniques used were essentially as described (9). Briefly, progeny

plasmids were assayed for mutant *supF* genes by transforming SY204 bacterial cells to ampicillin resistance and plating them on Luria-Bertani agar containing 5-bromo-4-chloro-3-indolyl β-D-galactosidase (X-gal) isopropyl β-D-thiogalactoside, and ampicillin. Cells containing plasmids with an inactive *supF* form light blue or white colonies, rather than dark blue colonies because the tRNA is unable to suppress the amber mutation in the bacterial β-galactosidase gene.

Mutant colonies were restreaked on these agar plates to confirm the phenotype, and plasmid was amplified and extracted, analyzed by agarose gel electrophoresis, and a portion was used to transform SY204 bacteria once more to ensure that the inability of the original transformants to utilize X-gal was the result of an inactivated *supF* gene. Mutants which showed no change in gel electrophoretic pattern were amplified and purified for DNA sequence analysis using either CsCl/ethidium bromide centrifugation as described previously (9) or using a small scale alkaline lysis procedure essentially as described by Zagursky et al. (24). Purified *supF* gene was sequenced as described (9), using the dideoxyribonucleotide method and ^{35}S-labeled adenosine 5′-[α-thio]triphosphate and the Klenow fragment of DNA polymerase I (New England Biolabs) or with modified T7 DNA polymerase (Sequenase, United States Biochemical).

Determination of Sites of Carcinogen-Induced Adducts. An estimation of the positions of AF adducts in the *supF* gene of carcinogen-treated plasmids was made using the in vitro DNA polymerase-stop assay of Moore and Strauss (25) essentially as described previously (10), except that the reactions were run not only with ^{35}S labeled adenosine-5′-[α-thio]-

triphosphate as in reference 10, but also with the pBR322 EcoRI site primer end-labeled with [γ^{32}P]-labeled adenosine 5'-triphosphate (NEG-034S and NEG-002Z; New England Nuclear, Boston, MA). Use of end-labeling eliminates the need to take into account the number of adenine bases incorporated during polymerization. Briefly, double-stranded plasmid containing 8.6 or 40.7 AF-adducts was denatured and annealed with the pBR322 EcoRI site primer. The length of the DNA from the primer site to the end of the *supF* gene is ~230 nucleotides. Therefore, the average number of adducts per strand of *supF* gene was 0.18 or 0.85. The polymerization reaction was carried out as described in reference 9 for the sequencing reaction, except that the dideoxynucleotides were omitted to allow the adducts to cause the termination. DNA from the four dideoxy sequencing reactions, carried out on an untreated template, was electrophoresed on the same gel to serve as DNA size markers. The relative intensities of the bands on the autoradiograph of the gel using [γ -^{32}P]-labeled adenosine 5'-triphosphate end-labeled primer were determined by a digital image analyzer (Visage 110, Bio Image, Kodak, Rochester, NY) using the whole band analysis software. This software provides band quantification by defining boundaries of bands based on inflection points and integrating the optical density of the whole band and presenting these data as percent of the total density of the material being analyzed in each lane of the gel.

RESULTS

Characterization of N-AcO-TFA-AF Treated Plasmids. pZ189 was treated with various concentrations of tritium-labeled N-AcO-TFA-AF and assayed for the number of residues bound per plasmid and for loss of ability to transform SY204 bacterial cells to ampicillin resistance. As shown in Figures 1A and 1B, the number of AF adducts per molecule of plasmid increased linearly with the concentration of N-AcO-TFA-AF used, and the transforming activity of modified plasmid decreased in direct proportion to the number of AF residues bound. About 36 AF residues bound were required to lower the transforming activity of the treated plasmid to 37% of the untreated control plasmid. Aliquots from samples of N-AcO-TFA-AF-treated DNA were analyzed by reverse-phase HPLC to determine the nature of the adducts formed. Two peaks of radioactivity were observed, a major peak at 28 min, and variable quantities of a minor peak which eluted at 19 min. Consistent with previous studies (18,26) the major peak corresponds to intact dG-C8-AF while the minor peak represents a ring opened form of this adduct (data not shown). No other adducts were seen.

Characterization of *supF* Mutants Induced by N-AcO-TFA-AF Treatment. Plasmids treated with various doses of N-AcO-TFA-AF or untreated plasmids were transfected into 293 cells and allowed to replicate. The progeny plasmids were rescued and assayed for the frequency of *supF* mutants by transforming the SY204 indicator bacteria. There was a linear increase in the frequency of *supF* mutations as a function of the number of AF adducts per plasmid (Figure 1C). At the highest level of AF adduct formation tested, i.e., 40.7 adducts per pZ189, the frequency of *supF* mutations was

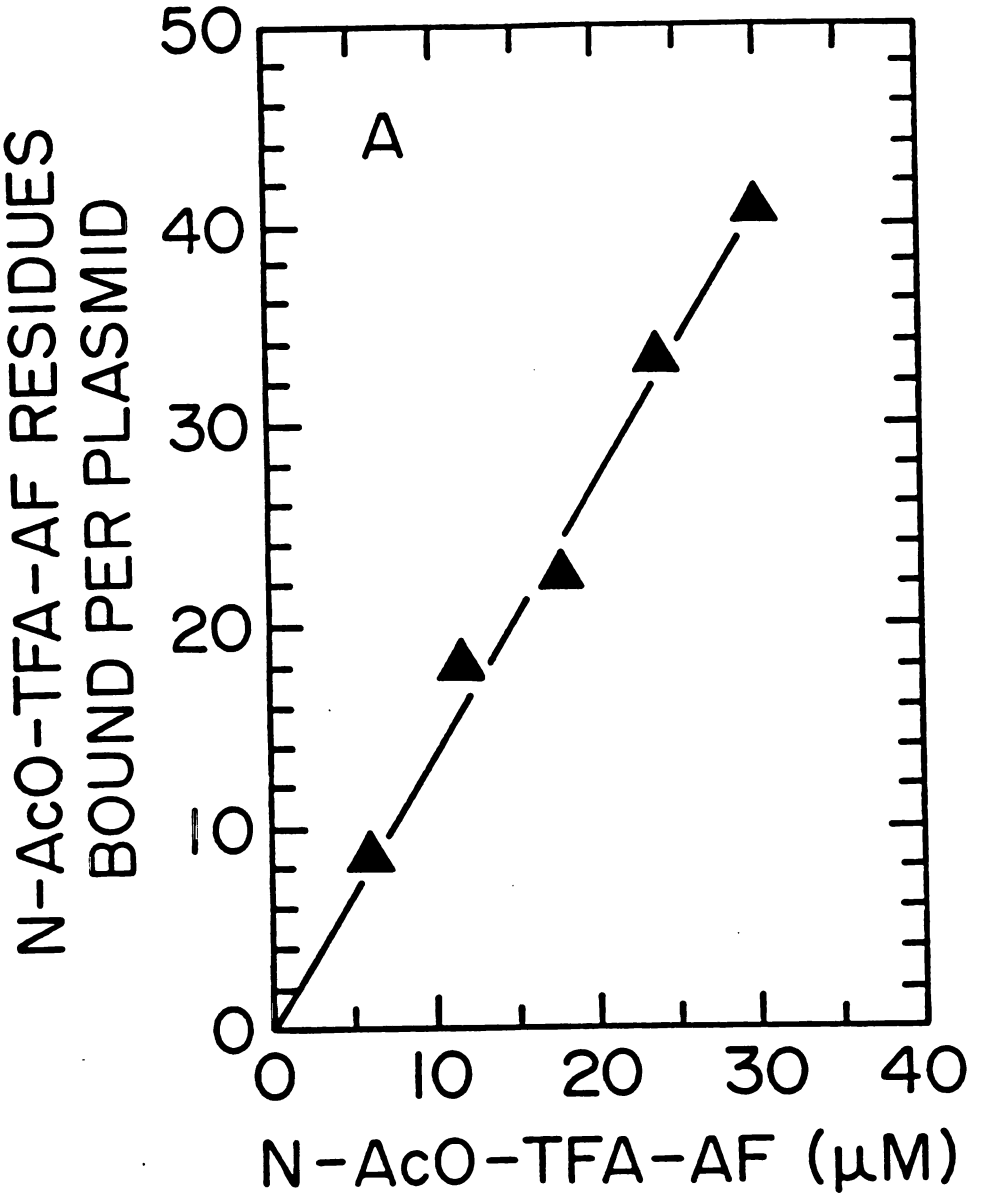

Figure 1. (A) Number of AF adducts bound per plasmid as a function of concentration of N-AcO-TFA-AF.

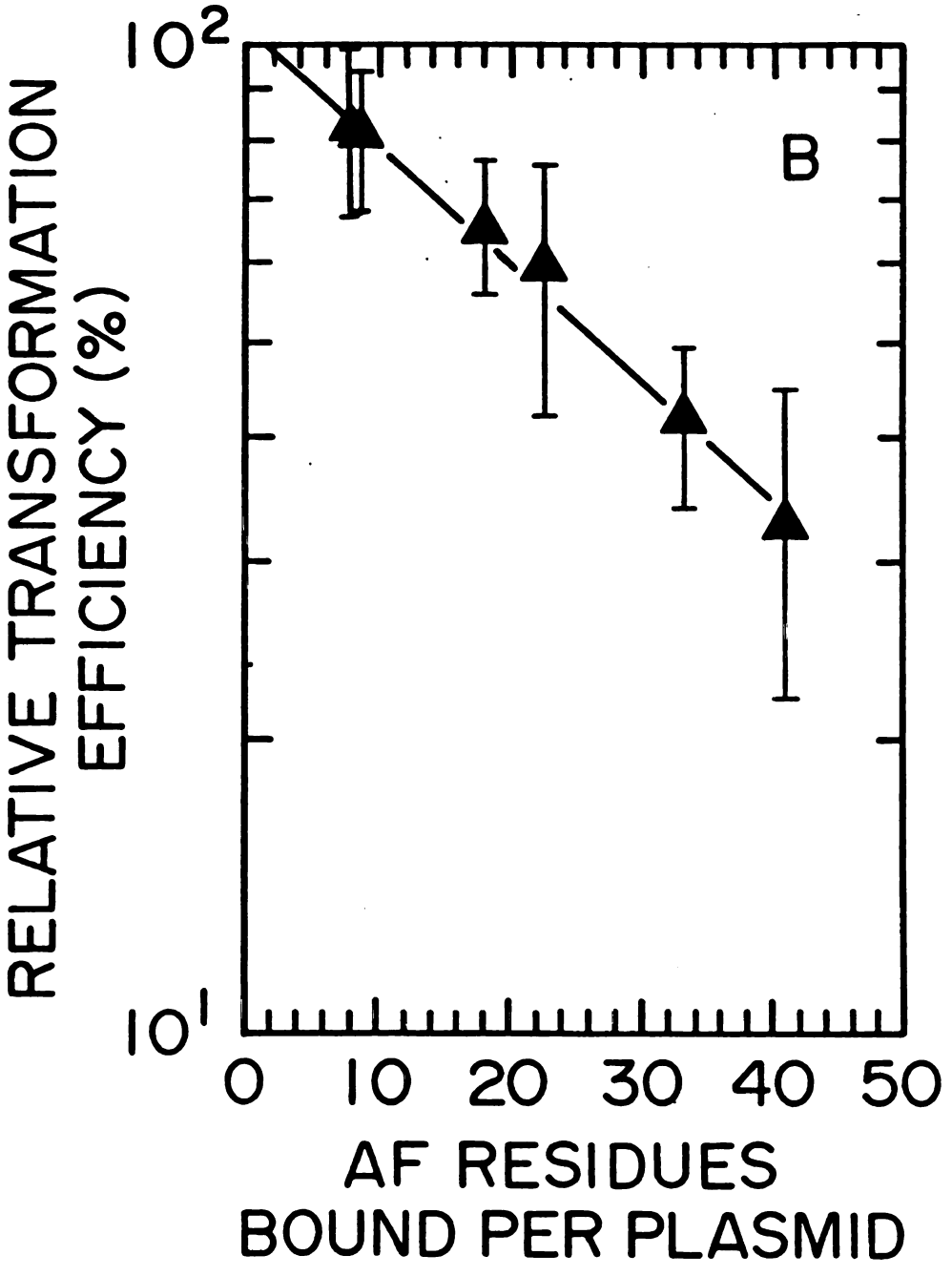

Figure 1. (B) Relative frequency of transformation of bacteria to ampicillin resistance as a function of the number of adducts per plasmid (Error bars indicate the standard errors of three determinations).

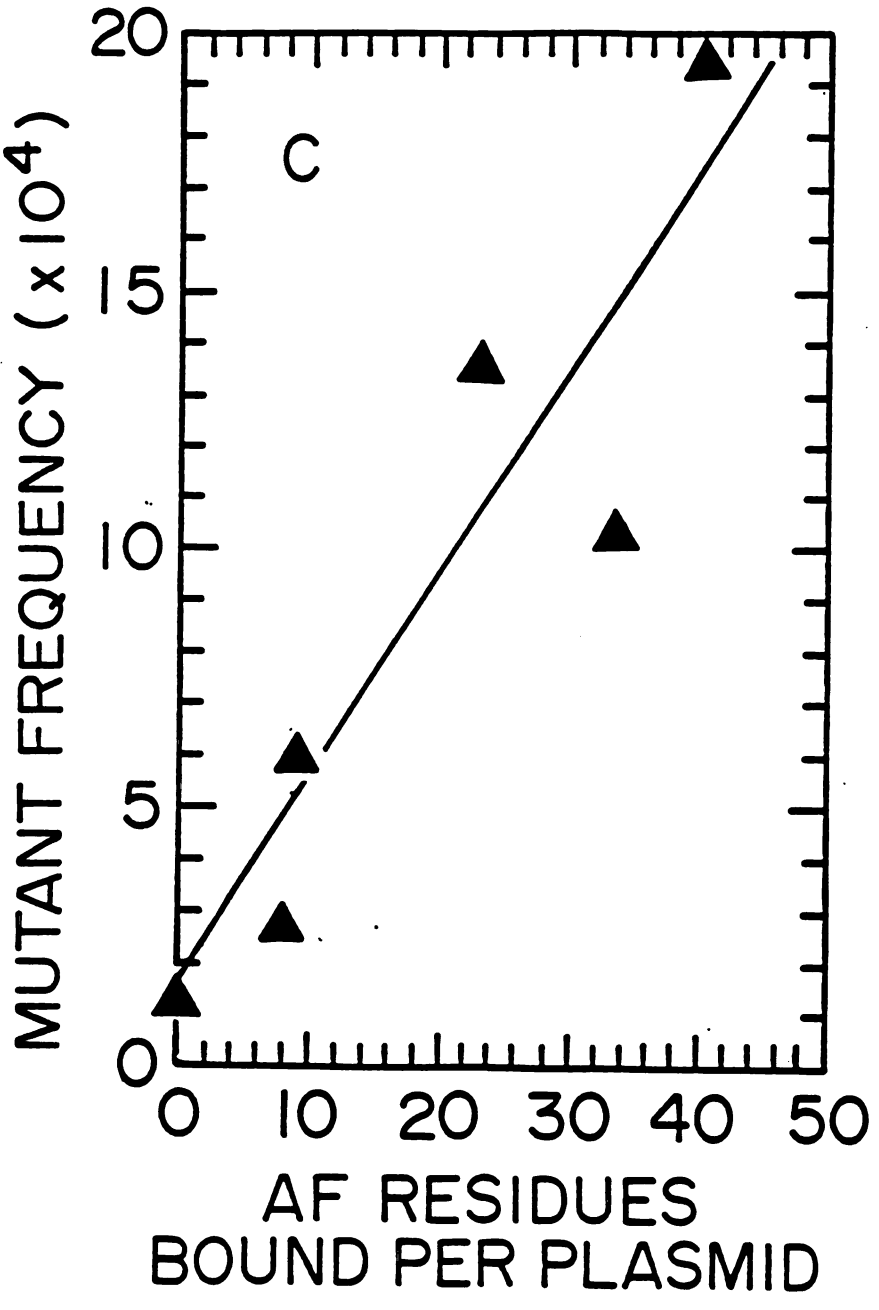

Figure 1. (C) Frequency of *supF* mutants as a function of the number of AF adducts per plasmid.

19.4, which is 15 times higher than the background frequency of 1.3×10^{-4}.

A total of 118 mutant plasmids derived from N-AcO-TFA-AF treated plasmids were analyzed for altered gel mobility to see if they contained gross alterations, i.e., deletions or insertions of >150 bp. Only 14 showed such alteration. The *supF* gene in the majority (47) of the 86 mutants derived from plasmids carrying 22.6, 33.2, or 40.7 adducts per plasmid that did not show altered gel mobility were further analyzed by DNA sequencing for small deletions or insertions and for point mutations, i.e., base substitutions or deletions or insertions of 1,2, or 3 bp. Table 1 columns 7, 8, and 9 summarize the results and compare them with the results obtained with untreated plasmids and with plasmids carrying 8 or 9 adducts. At least 63% of the spontaneous background mutants exhibited altered gel mobility or contained deletions or insertions of 9 bp or more. In contrast, of the 86 mutants obtained with plasmids carrying the three highest levels of adducts that were analyzed for altered gel mobility, only 4 (4.6%) showed altered gel mobility (Table 1). Since not all the mutants which appeared to contain point mutations were sequenced, it is possible that a few of those that were not sequenced represent siblings. If this is the case, the freuqencies of mutants containing point mutations (column 10) are slightly overestimated (<10%). However, the presence of siblings does not affect the frequency of *supF* mutants (column 4) since siblings contribute equally to both numerator and denominator.

The specific locations of the point mutations derived from 50 N-AcO-TFA-AF treated plasmids are shown in Fig 2. There were four prominent hot

Table I. Analysis of mutants obtained by transformation of E. coli with progeny of N-AcO-TFA-AF-treated pZ189 generated during replication in 293 cells

Adducts per plasmid	No. of human cell transfection experiments	*supF* mutants[a]/ transformants	Frequency of *supF* mutants (10^4)	Plasmids with altered gel mobility[b]/no. examined	Total plasmid *supF* genes sequenced	Characterization of sequenced mutants			Freq. of mutants with point mutations (10^4)[f]
						No. with deletions[c]	No. with insertions[d]	No. with point mutations[e]	
0	25	42/313753[g]	1.3	8/40	26	14	3	9	0.3
8	1	6/23053	2.6	0/4	1	0	0	1	--
8.6	2	30/51610	5.8	5/24	2	0	0	2	--
22.6	3	38/27740	13.7	2/29	19	2	0	17	11.1
33.2	3	25/22640	11.0	1/20	10	0	1	9	9.0
40.7	3	44/22696	19.4	1/41	18	1	0	17	17.4

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

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

[c]Deletion of 9 to 150 bp.

[d]Insertion of 10 to 30 bp.

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

[f]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 9 divided by that in column 6 plus those mutants showing altered gel mobility (numerator, column 5).

[g]These data for the spontaneous mutants include previously published results obtained with untreated plasmids.

spots, positions 123, 133, 159, and 169. Position 123 was also shown to be a hot spot for BPDE (9) and 1-NOP (10), but not for (-)BcPhDE-2 (16). Position 133 is a hot spot for BPDE and (-)BcPhDE-2; 159 is a hot spot for 1-NOP; but position 169, at which 16% of the mutations occurred and at which 9% of the dG-C8-AF adducts were located, is a hot spot unique to *N*-AcO-TFA-AF-treated plasmids since it was not a hot spot for any other structurally-related chemical carcinogen.

Table 2 shows the kinds of point mutations induced by dG-C8-AF, and compares the data with that obtained with spontaneous mutants. In analyzing the frequency of specific kinds of mutations, only unequivocally independent mutants were included, i.e., those that carried a unique alteration or were derived from independent sets of human cell transfection experiments. Only 14% (3/22) of the mutants analyzed from untreated plasmids carried single base substitutions, while 64% (14/22) contained deletions or insertions of more than 2 bp. In contrast, 84% (42/50) of the mutants analyzed from the treated plasmids showed single base substitution mutations and only 8% (4/50) showed deletions or insertions of more than 2 bp. Table 3 gives the data on the specific types of base substitutions. All of the 49 independent base substitution mutations obtained with N-AcO-TFA-AF-treated involved plasmids G·C pairs. 82% (40/49) were transversions, 66% (32/49) were G·C → T·A.

Correlation between Sites of AF-Adducts and Locations of AF-Induced Mutations. To determine if the four hot spots for mutation corresponded to hot spots for formation of dG-C8-AF adducts, we carried out the polymerase-stop assay using the Klenow fragment of *E. coli* polymerase I. This method of estimating the percentage of carcinogen adducts formed at

Figure 2. 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 *Eco*RI rightward primer. The point mutations observed in the progeny of N-AcO-TFA-AF-treated plasmids are placed below the sequence. The rectangle represents a deleted guanine. Every 10th residue and the anticodon triplet are underlined.

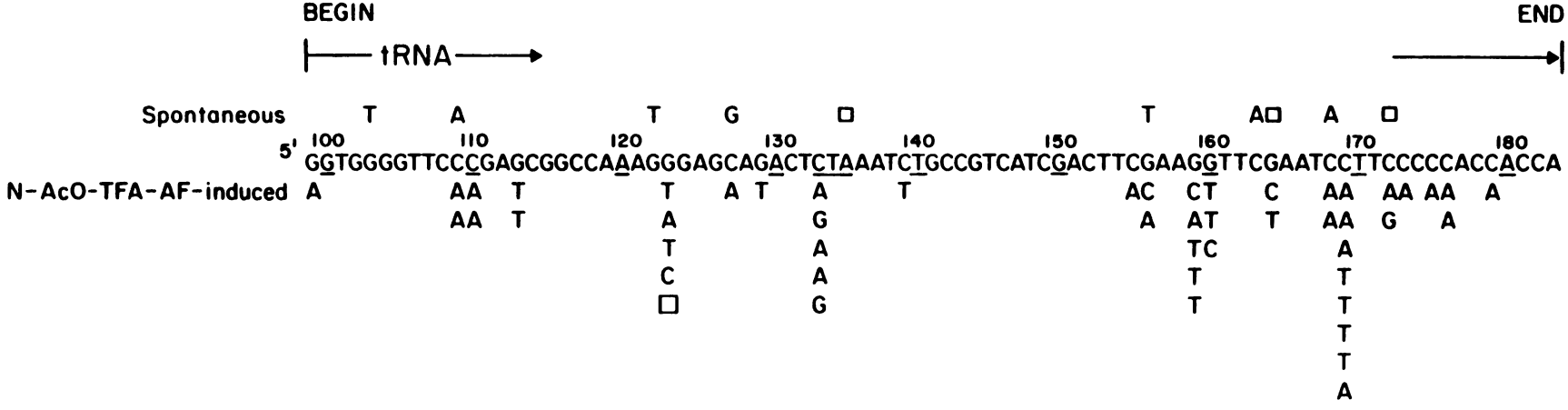
BEGIN
END
tRNA
Spontaneous
5'
N-AcO-TFA-AF-induced
100
110
120
130
140
150
160
170
180
GGTGGGGTTCCCGAGCGGCCAAAGGGAGCAGACTCTAAATCTGCCGTCATCGACTTCGAAGGTTCGAATCCTTCCCCCACCACCA

Figure 2

Table II. Analysis of Sequence Alterations Generated in the *supF* gene by replication of N-AcO-TFA-AF-treated or untreated pZ189 in 293 cells

Sequence Alterations	No. of times occurring	
	Untreated	N-AcO-TFA-AF-treated
Single base substitution	3	42
Two base substitutions		
Tandem	0	1
≤ 20 bases apart	2	0
> 20 bases apart	0	1
Three Base substitutions		
≥ 20 bases apart	0	1
Deletions		
Single G•C pair	2	1
Single A•T pair	1	0
Tandem base pairs	0	0
4-20 bp	4	2
> 20 bp	8	1
Insertions		
4-20 bp	2	0
> 20 bp	0	1
Total Sequenced	22	50

Table III. Kinds of base substitutions generated by replication of N-AcO-TFA-AF-treated or untreated pZ189 in 293 cells

Base Change		No. of mutations observed	
		Untreated	N-AcO-TFA-AF-treated
Transversions			
G•C	T•A	6	32
G•C	C•G	1	8
A•T	T•A	0	0
A•T	C•G	0	0
Transitions			
G•C	A•T	0	9
A•T	G•C	0	0
	Total	7	49

particular sites on the gene relies on two assumptions: that the density of the bands in a sequencing gel is proportional to the number of DNA molecules of a particular length and that the length of the fragments reflects the chance of adduct-induced premature termination of the polymerization. Using this *in vitro* replication assay and a gapped double-stranded M13 template containing approximately six dG-C8-AF or dG-C8-AAF adducts in the intact strand, Strauss and his colleagues (27) showed that the latter adduct was equally effective in terminating polymerization by Sequenase and by the Klenow fragment of E. coli polymerase I. However, dG-C8-AF was much more effective in terminating polymerization by the Klenow fragment than by Sequenase. These investigators also showed that with dG-C8-AF, termination of polymerization by the Klenow fragment occurred either directly opposite the guanine or one base prior to it. This characteristic does not interfere with quantitating the frequency of termination at guanine except where the guanine adduct occurs in a run of G's.

Figure 3 shows a diagram of the relative intensities of the termination sites as judged from the intensities of the bands on the gel. Every G in the sequence analyzed exhibited a band. 10 of the 26 G's in the sequence analyzed were single; there were four doublets, 1 triplet, and a run of 5 G's. For the G's that were not single, a band directly opposite the final G of a run and a band one base prior to the first G give unequivocal information. The density of the band for the first G in a doublet, or for all but the final G of a run was divided appropriately (i.e., by two or by three) and the value was assigned to the appropriate position. When these "binding" data were analyzed for correlation with

Figure 3. Relative frequency of AF adducts in the 3′ to 5′ strand of the coding region of the *supF* gene, as judged by the polymerase-stop assay, and location of N-AcO-TFA-AF induced base substitutions in the corresponding 5′ to 3′ strand. The Klenow fragment of DNA polymerase I was used with the *Eco*RI rightward sequencing primer to determine polymerase-stop sites (panel shown above the coding sequence of the *supF* gene). The relative intensities of the bands on the autoradiograph were determined by digital image analyzer. The data, expressed as percent of the total band densities, are represented by the lengths of the lines. The base substitutions shown below the *supF* tRNA gene are the cytosine base changes found in the corresponding 5′ to 3′ strand. Every tenth base is underlined.

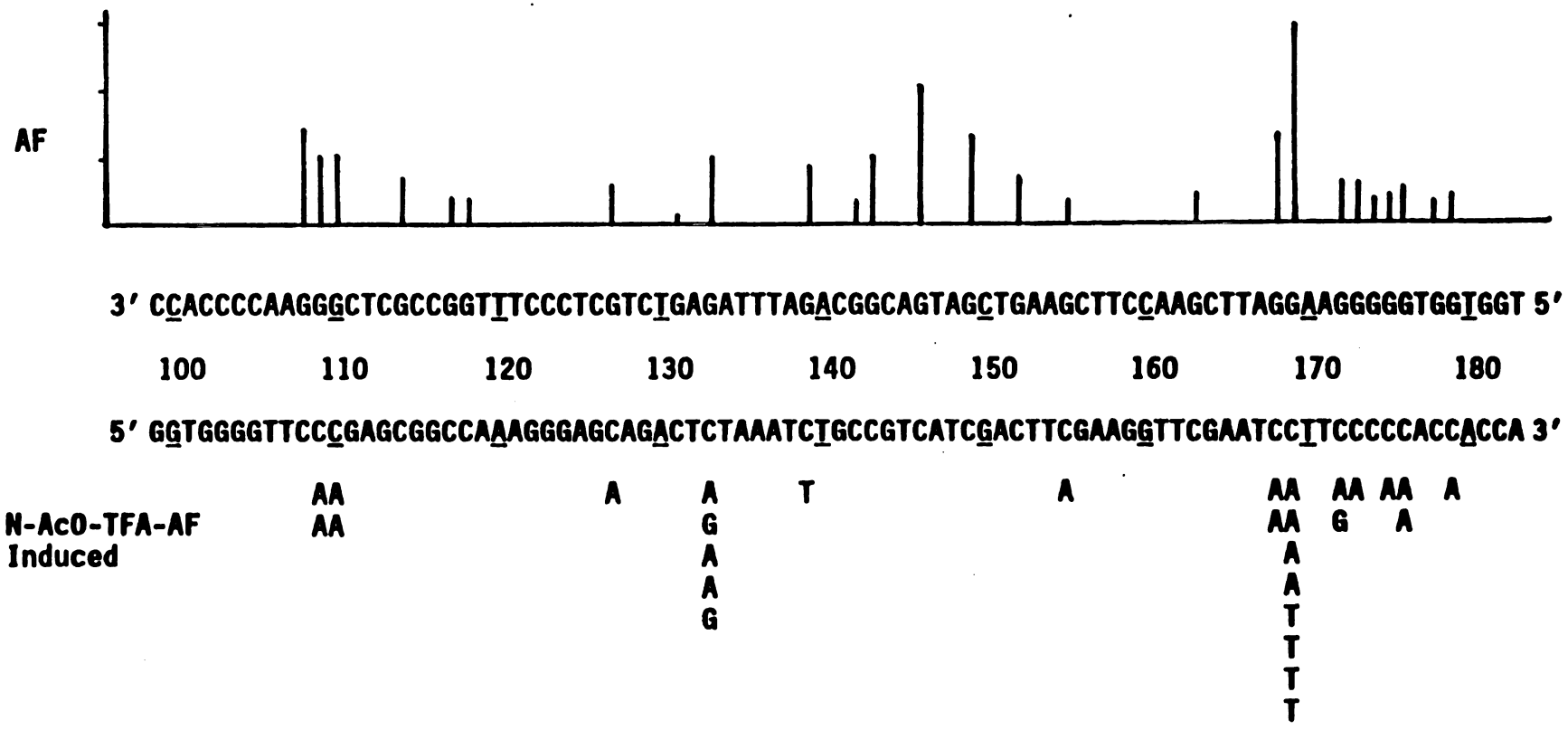
AF
3′ CCACCCCAAGGGCTCGCCGGTTTCCCTCGTCTGAGATTTAGACGGCAGTAGCTGAAGCTTCCAAGCTTAGGAAGGGGGGTGGTGGT 5′
100
110
120
130
140
150
160
170
180
5′ GGTGGGGTTCCCGAGCGGCCAAAGGGAGCAGACTCTAAATCTGCCGTCATCGACTTCGAAGGTTCGAATCCTTCCCCCACCACCA 3′
N-AcO-TFA-AF Induced
AA
AA
A
A
G
A
A
G
T
A
AA
AA
A
A
T
T
T
T
AA
G
AA
A
A

Figure 3

mutational hot spots (see below), there was ambiguity only in the case of positions 168 and 169. Even if we had not split the difference in band density, the two G's at positions 168 and 169 would have represented hot spots for binding, so there is no ambiguity about the correlation between binding and mutations at the latter position.

Figure 4 shows an example of a polyacrylamide gel showing the site of termination on a template containing 41 adducts per plasmid (0.85 adducts per strand of *supF*). The bands correspond to position one nucleotide prior to or directly opposite every cytosine in the DNA sequencing standard lane indicating that DNA synthesis terminated at either of these sites in the template. No bands corresponding to positions one nucleotide away from or opposite any base other than guanine were seen, and there was no evidence of any interference with polymerization when untreated plasmid was used (Figure 4, lane 0). As shown in Figure 3, the relative frequency of DNA adducts, as estimated from the frequency of termination, ranged from 0.7% (position 131) to 9% (position 169). In some cases there was good correlation between the extent of binding and the frequency of mutation induction. For example, position 169, the strongest mutation hot spot for N-AcO-TFA-AF (16%) showed the highest frequency of adduct binding (9%); position 155, which showed a low frequency of mutations (2%) also showed very little binding (1.4%). However, position 108 showed relatively high binding frequency (4.2%), but no mutations were observed at that site. Positions 114, 133, 139, and 143 all showed medium mutagen binding (~3.0%), but 133 is a mutational hot spot (10%), whereas 114, 139, and 143 are very cold sites for mutations (0 - 2%). Some of the lack of correlation between the

Figure 4. Polyacrylamide gel showing the sites of termination in the *supF* gene of the Klenow fragment of *E. coli* polymerase I on a template containing 0.85 dG-C8-AF adducts per 230 bases by using the *in vitro* DNA polymerase-stop assay of Moore and Strauss (25), as described in reference 10. The GTAC lanes are dideoxy sequence standards obtained by incubating untreated DNA templates with the Klenow fragment and dideoxyribonucleotides. Lane 41 shows the product of the stop assay carried out on a template prepared from N-AcO-TFA-AF treated pZ189 containing 40.7 AF adducts per plasmid; lane 0 represents a template from untreated plasmid. The stop assay reactions for the templates shown here were run using ^{35}S-labeled adenosine 5′-[α-thio] triphosphate. The reaction was chased with unlabeled dATP for 15 min. The numbers on the left identify the location of bases in the *supF* gene.

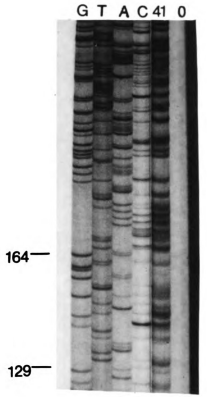

Figure 4

results in Figure 2 and Figure 3 can be explained by the so called "silent mutations". For example, positions 146 and 149 showed high frequency of adduct binding (>4%) and yet no mutations, but the cumulative data from studies with the *supF* tRNA gene (12) demonstrate that these two positions have only been found to cause a phenotypic change when both positions were changed.

DISCUSSION

The model hepatic carcinogen, 2-acetylaminofluorene (AAF), and its metabolites have been studied intensively for many years. It has been shown that when AAF or its *N*-hydroxy derivative is administered to rats, the primary adduct formed in the DNA of the mammary gland or the liver is dG-C8-AF (100% in mammary tissue, 80% in liver) (28-30). Formation of dG-C8-AF is thought to result from the metabolic production of *N*-acetoxy-AF (N-AcO-AF) by cytosolic or microsomal enzymes (31-32). Tumor induction in the rat mammary gland has been related to the cytosolic activation of *N*-hydroxy-AAF by N,O-acyltransfer (33) to yield N-AcO-AF, the reactive derivative formed on solvolysis of N-AcO-TFA-AF (34). The acetylated form, dG-C8-AAF, represents ~15% of the total DNA adducts in rat liver (35) and a still more minor acetylated adduct (<5%) is formed at the N2 position of guanine (36). These acetylated adducts are formed primarily by conjugation of N-OH-AAF with sulfate in rat liver (38). But when bacteria or mammalian cells in culture are treated with N-AcO-AAF or N-OH-AAF, the dG-C8-AF adduct is the principal adduct formed (17,21, 39). The mutagenic action of N-AcO-AAF or N-OH-AAF in bacteria (39,40), human cells (40,41), and other mammalian cells (19,21,42), is well-documented. In addition, a number of studies of the kinds of mutations induced by dG-C8-AF or dG-C8-AAF located randomly in plasmid or viral DNA (43-45) or located at specific sites (46-49) have recently been carried out.

The present study was undertaken to compare the frequency and specific kinds of mutations induced by dG-C8-AF adducts when pZ189 replicates in 293 cells with what is already known from other systems

about mutations induced by dG-C8-AF or dG-C8-AAF and with what has been found previously in the *supF* gene when plasmids treated with structurally-related carcinogens, such as BPDE and 1-NOP, replicate in these same human cells (9,10,12). Such comparative studies can shed light on the molecular and cellular mechanisms by which carcinogen-induced mutations arise.

We conclude from our results that the mutations obtained with the N-AcO-TFA-AF-treated plasmids were targeted to the dG-C8-AF adducts. Several reasons support this conclusion: (a) the increase in frequency of mutants was linearly correlated with the number of adducts per plasmid; (b) when the mutants obtained from the plasmids carrying 22.6, 33.2 or 40.7 adducts were analyzed, the frequency of those containing gross rearrangements, or deletions or insertions greater than 1 bp (i.e., 4/27740, 2/22640, and 2/22696, respectively), was equal to or less than the background frequency of 1.3×10^{-4}; the rest carried point mutations involving G•C base pairs, (c) 84% of the 50 mutants sequenced contained only a single G•C base substitution, one contained a single deletion of a G•C bp (a minus one frameshift) (d) none of the mutations in the 5′ to 3′ strand were located at sites where termination of polymerization on a 3′ to 5′ template by the Klenow fragment was not detected, (e) the two mutational hot spots for which the dG-C8-AF adduct would be located in the 3′ to 5′ strand (positions 133 and 169) were also prominent positions for termination of polymerization.

Loeb and Preston (50) have suggested that transversion mutations result from apurinic or apyrimidine sites in DNA into which an adenosine triphosphate is inserted during replication, rather than from replication past a non-instructive base adduct (trans-lesion synthesis). The majority

(66%) of the base substitutions we observed with N-AcO-TFA-AF-treated plasmids were G·C → T·A transversions. This may have resulted from a DNA polymerase preferentially inserting adenosine triphosphate opposite the dG-C8-AF adduct, as suggested by Strauss et al. (51). However, Norman et al. (52) recently found that an interpretable NMR spectrum could be obtained from a double-stranded oligonucleotide containing a single dG-C8-AF adduct with adenine opposite the modified guanine, whereas in the particular oligonucleotide used for their analysis, the spectrum for cytosine opposite the modified guanine was not interpretable. If an adenosine triphosphate inserted opposite dG-C8-AF during replication of pZ189 in the 293 cells were to be accommodated in the same way, this would result in the type of transversions that we observed most frequently, i.e., G·C → T·A. The G·C → C·G and G·C → A·T base changes could reflect less stable mispairing with guanine or thymine nucleotides opposite the modified guanine. This hypothesis is supported by the finding by Sahm et al. (27) that the modified T7 polymerase, Sequenase, can sometimes replicate past dG-C8-AF adducts in double-stranded gapped M13 molecules, but can do so much less frequently in the case of dG-C8-AAF adducts.

Our results with pZ189, showing that 98% of the dG-C8-AF-induced mutations (49/50) consisted of G·C base substitutions, agree with what was recently found by Carothers et al. (36) in an endogenous gene of CHO cells treated with N-AcO-AAF. In their study, 88% of the adducts were dG-C8-AF. Of the 18 base substitution mutations they obtained, 75% were G·C → T·A transversions. Bichara and Fuchs (39) also found that base substitutions were the predominant mutation seen when a plasmid carrying randomly-located dG-C8-AF adducts replicated in *E. coli* bacterial cells.

Romano et al. (42) found this using a plasmid containing a site specific dG-C8-AF adduct replicating in bacteria. Gupta et al. (40) also found that the majority of the mutations induced by the dG-C8-AF adduct randomly located in single stranded or double stranded M13 virus replicating in *E. coli* were base substitutions, but other mutational changes were also found.

Both N-AcO-TFA-AF- and 1-NOP-treated plasmids contain adducts at the C8 position of guanine and the covalently bound residues (aminofluorene, AF and aminopyrene, AP) are structurally quite similar. Although the dG-C8-AF adduct was not as effective as the dG-C8-AP adduct in interfering with bacterial transformation to ampicillin resistance (cf. Fig. 1B with Fig. 1B of ref. 10), the frequency of *supF* mutants induced by these adducts was very similar (cf. Fig. 1C with Fig. 1C of ref. 10) and the kinds of mutations induced were very similar. 70% of the G•C base substitutions induced with 1-NOP and 66% of those induced with N-AcO-TFA-AF were G•C → T•A transversions, but the location of the base substitution mutations in the *supF* gene was not identical. Although N-AcO-TFA-AF- and 1-NOP-treated plasmids gave two prominent hot spots in common (at positions 123 and 159), each carcinogen produced its own set of unique hot spots, which also differed from the set found with BPDE (9) or (-)BcPhDE-2 (16).

Our results showing that the mutation hot spots with dG-C8-AF did not correlate exactly with hot spots for adduct formation, as estimated from the frequency of termination of polymerization by the Klenow fragment, are similar to the results of Yang et al. with 1-NOP and BPDE (10). One possible explanation for the lack of correlation is that

excision repair by the host cells plays a role and preferentially removes adducts from some sites in the plasmid faster than from other sites. Fuchs and his coworkers (43) coined the phrase "mutation-prone sequences" to explain the lack of correlation they observed between the sites of AAF-induced frameshift mutations in a bacterial plasmid and the site at which T4 DNA polymerase exonuclease IV activity was blocked by AAF residues and Brash et al. (53) proposed a similar "pass/fail" model to explain the lack of correlation of mutation frequency with sites of UV-induced photoproducts in the *supF* gene when UV-irradiated pZ189 replicated in simian virus 40-transformed human cells. The results of these groups, as well as the data from our laboratory, suggest that neighboring bases may play a role in determining whether a particular adduct or photoproduct gets converted into a mutation.

In the present study of N-AcO-TFA-AF, we observed very few multiple mutations occurring in a single mutant plasmid, following replication in 293 cells (only 3 of 50, Table 2). Yang et al. (10) also found this for 1-NOP- and BPDE-induced mutations. These results contrast with what Bredberg et al. (54) found with UV-irradiated pZ189 replicating in SV40 transformed human cells, and Hauser et al. (55) found when UV-irradiated pZ189 replicated in an SV40-transformed monkey cell line. The reason for this difference is currently under investigation.

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CHAPTER III

Acetylaminofluorene Adducts Primarily Cause Base Substitutions When a Shuttle Vector Replicates in Human Cells

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Summary

2-Acetylaminofluorene, a well studied liver carcinogen, forms two major DNA adducts, viz., *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-C8-AAF), and its deacetylated derivative *N*-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF). To investigate the mechanisms of mutagenesis by this carcinogen, we determined the frequency and kinds of mutations induced when a shuttle vector containing dG-C8-AAF adducts replicated in human cells and compared this spectrum with that which we found previously with dG-C8-AF (Mah *et al.*, *Carcinogenesis*, 10: 2321, 1989). The shuttle vector, pZ189, carrying the *supF* tRNA gene as the target for mutations, was treated with radiolabeled *N*-acetoxy-2-acetylaminofluorene to obtain the acetylated adducts at the C-8 position of guanine, and allowed to replicate in the human kidney cell line 293. The progeny plasmids were examined for the frequency and spectrum of mutations induced. The frequency of *supF* mutants increased with dose and number of dG-C8-AAF adducts. When compared on the basis of the number of adducts per plasmid, dG-C8-AAF was two times more mutagenic than dG-C8-AF, and each induced mainly base substitutions (85%), predominantly G·C --> T·A transversions. However, the mutations were not randomly distributed, and the locations of "hot spots" and "cold spots" for mutations were unique for each agent. There was good correlation between the sites of dG-C8-AAF formation, as determined by the DNA polymerase stop assay, and the sites of mutations. This indicates that at least in part the occurrence of mutational "hot spots" and "cold spots" reflects the preferential binding of the carcinogen to certain sites. However, excision of adducts from particular

sites in the target gene before replication occurs may also play a role in determining the mutation spectrum.

1. Introduction

Aromatic amines are valuable chemicals in many areas of industry and research and are particularly important as intermediates in the formation of dyes and pharmaceuticals. A number of aromatic amines are known to produce cancer in experimental animals and in humans (Wilson *et al.*, 1941; Miller, 1978; Kriek, 1979; Garner *et al.*, 1984). One of the best studied model aromatic amine used to investigate the mechanisms of carcinogenesis is 2-acetylaminofluorene (AAF), a strong liver carcinogen in male rats. To be carcinogenic AAF must undergo conversion to reactive metabolites that can interact with the macromolecules of the target tissue (reviewed by Miller, 1978). The reactive metabolites bind to DNA, producing two major adducts : N-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-C8-AAF) and its deacetylated form, N-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF) (reviewed by Beland & Kadlubar, 1990). These two adducts appear to be removed from DNA at different rates. The acetylated dG-C8-AAF adduct is rapidly removed from rat liver in vivo, with a half-life of approximately 7 days (Kriek, 1972). This adduct was also shown to be rapidly removed from primary cultures of rat hepatocytes with a half-life of approximately 10 hr (Howard *et al.*, 1981). In contrast, dG-C8-AF adducts have a half-life of >40 hr in primary rat hepatocyte cultures (Howard *et al.*, 1981), and significant quantities of this adduct have been shown to persist in liver cells for up to one month after discontinuation of AAF diet (Poirier *et al.*, 1984). Even so, no simple correlation between persistence of an adduct and tumor induction has been established, since dG-C8-AF was also found to persist in rat kidney and female rat liver

which are tissues resistant to *N*-hydroxy-2-acetylaminofluorene (N-OH-AAF)-induced tumorigenesis (Beland *et al.* 1982).

Physico-chemical studies with dG-C8-AAF and dG-C8-AF adducts reveal a significant difference in the way they alter the conformation of DNA. Fuchs and his colleagues have proposed the "insertion-denaturation" model for dG-C8-AAF, in which an AAF adduct triggers a major local distortion of the DNA helix by inducing the modified guanine to rotate around the *N*-glycosylic bond to assume the *syn* conformation, stacking the fluorene moiety between the bases, and resulting in a local denaturation involving 12 base pairs (Fuchs & Daune, 1972; Fuchs & Daune, 1974; Fuchs, 1975). Grunberger and coworkers (1970) also observed such conformational changes, and they suggested a similar "base displacement" model for dG-C8-AAF (Levine *et al.*, 1974). In contrast, dG-C8-AF adducts retain their normal orientation without causing a major change in the B-DNA structure (Evans *et al.*, 1980; Santella *et al.*, 1980). Circular dichroism and proton magnetic resonance studies of dApdG modified with AAF and AF residues (Santella *et al.* 1980), as well as minimized potential energy calculations (Broyde & Hingerty, 1983), support these models.

In vitro DNA polymerization studies by Strauss and his coworkers involving single stranded bacteriophage DNA containing dG-C8-AAF or dG-C8-AF indicate that these adducts differ in their ability to interfere with polymerization. The dG-C8-AAF residue present in the DNA template causes T4 DNA polymerase to terminate polymerization one nucleotide before the site of the adduct, whereas, the deacetylated residue induces termination of DNA synthesis after incorporation of nucleotide opposite the modified guanine (Moore & Strauss, 1979; Moore *et al.*, 1982). dG-C8-

AAF also causes termination of polymerization by modified T7 DNA polymerase (Sequenase) one nucleotide before the adduct, whereas, the same enzyme appears to be able to bypass dG-C8-AF lesions frequently (Sahm *et al.*, 1989). The results of these *in vitro* studies suggest that there could be a significant difference in the kinds of mutations induced by these two adducts and, perhaps, in their location in the target gene.

To examine the mechanism of mutagenesis by these two different adducts, Fuchs and his colleagues carried out a comparative study using the tetracycline-resistance gene in the plasmid pBR322 as target for mutagenesis. The plasmids were treated with *N*-acetoxy-2-acetylaminofluorene (N-AcO-AAF) or *N*-hydroxy-2-aminofluorene (N-OH-AF) to obtain dG-C8-AAF and dG-C8-AF adducts, respectively, and then were allowed to replicate in *Escherichia coli*. These investigators found significant differences in the kinds of mutations induced by these adducts. While dG-C8-AAF were shown to induce almost exclusively frameshifts, dG-C8-AF caused mainly base substitutions, predominantly G·C --> T·A transversions (Koffel-Schwartz *et al.*, 1984; Bichara & Fuchs, 1985).

However, no one has carried out such a comparative study of mutations in mammalian cells. This is because when mammalian cells in culture are exposed to reactive metabolites of AAF, e.g., N-AcO-AAF, approximately 90% of the adducts formed are the deacetylated dG-C8-AF (Carothers *et al.*, 1986; Heflich *et al.*, 1988). Therefore, we made use of a shuttle vector reacted *in vitro* with N-AcO-AAF or *N*-acetoxy-*N*-trifluoroacetyl-2-aminofluorene (N-AcO-TFA-AF) to obtain dG-C8-AAF and dG-C8-AF respectively, and introduced the DNA into human cells to be replicated by the human polymerases. The study with the latter carcinogen

has been published (Mah *et al.*, 1989). The plasmid pZ189 (Seidman *et al.* 1985) carries a tyrosine suppressor tRNA (*supF*) gene as the target for mutations. The host cells we used are the human kidney 293 cell line. After replication, the progeny plasmids are rescued and assayed for mutations in indicator bacteria. The use of the *supF* gene, which codes for a tRNA, as the target gene for these mutation studies offers several advantages. Since the structure of the tRNA is essential for its purpose, almost any single base pair substitution, insertions or deletions within the *supF* structural gene will inactivate its function (Kraemer & Seidman, 1989). Therefore almost every mutation results in a phenotypic change. Another advantage is that the small size of the *supF* gene makes it easy to analyze at the sequence level and ideal for determining "hot spots" and "cold spots" for mutation induction by carcinogens, i.e., sites where there is a non-random distribution of mutations. It also allows us to use interference with polymerization as an assay to estimate the amount of adduct formation at the various sites. The use of *supF* as the target and human 293 cells as the host also makes it possible to compare the spectra of mutations induced in the same system by the structurally-related carcinogens 1-nitropyrene (1-NP) (Yang *et al.* 1988), 1,6-dinitropyrene (1,6-DNP) (Boldt *et al.* 1991), (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) (Yang *et al.* 1987), and benzo(c) phenanthrene (4*R*,3*S*)-dihydrodiol (2*S*,1*R*)-epoxide (-)-BGPhDE-2) (Bigger *et al.* 1989), to get some clues into mechanisms by which these carcinogens cause mutations.

Our results with dG-C8-AF adducts in this shuttle vector assay (Mah *et al.*, 1989) were similar to those found by Carothers *et al.* (1989) who

studied the mutations induced by dG-C8-AF adducts in the endogenous dihydrofolate reductase (*dhfr*) gene of Chinese hamster ovary (CHO) cells. The kinds of mutations induced by BPDE in the *supF* gene (Yang *et al.*, 1987) and in the endogenous hypoxantine (guanine) phosphoribosyltransferase gene of human fibroblasts were also very similar (Yang *et al.*, 1991). This confirms the validity of the use of the shuttle vector system as a simpler and quicker method of studying mutational spectra in mammalian cells. In the present paper we report the spectrum of mutations induced in the *supF* gene by the structurally related dG-C8-AAF adducts and compare them to the results of our earlier study with dG-C8-AF. When compared in terms of the number of adducts per plasmid, dG-C8-AAF was twice as mutagenic as dG-C8-AF. In both cases 85% of the induced mutations consisted of base substitutions, mainly G·C --> T·A transversions. However, each carcinogen induced its own spectrum of mutations. Unlike the results shown by Fuchs and coworkers (Fuchs *et al.* 1981; Koffel-Schwartz *et al.* 1984) in which > 90% of the mutations induced by AAF adducts when a plasmid replicated in *E. coli* were frameshift mutations, only 8% of the mutations induced by AAF adducts in our assay with human cells consisted of frameshifts. There was a good degree of correlation between carcinogen binding and mutation induction.

2. Materials and Methods

(a) *Cells and plasmid*

The human cell line used, 293, and the *E. coli* bacterial strain, SY204, as well as the shuttle vector, pZ189, carrying the *supF* gene and the gene for ampicillin resistance have been described previously (Yang *et al.*, 1987).

(b) *Formation of AAF adducts on the plasmid*

Purified plasmid DNA dissolved in 2 mM sodium citrate buffer, pH 7.0 at 1 mg/ml was added to a freshly prepared ethanol solution of generally-tritiated N-AcO-AAF (63 mCi/mmol) and incubated at 37°C for 30 min. To remove unreacted carcinogen, the DNA was subjected to repeated ether extractions, followed by purification with phenol buffered to pH 7.0 with sodium citrate. It was precipitated with ethanol and redissolved in sodium citrate buffer pH 7.0 and stored at -20°C until used.

(c) *Transfection and rescue of replicated plasmids*

The human host cells were prepared and transfected with plasmids as described (Yang *et al.* 1987). A series of individual dishes containing 2 to 3 X 10^6 cells/dish were used and after 48 h, the replicated plasmids were recovered and purified as described (Yang *et al.* 1987). In order to be able to distinguish between mutations that occurred frequently and putative siblings arising from within the same population, the plasmids derived from individual dishes were assayed separately for mutant *supF* genes.

(d) *Bacterial transformation and mutant characterization*

The techniques used were described by Yang *et al.* (1987). SY204 cells are sensitive to ampicillin and because of an amber mutation are unable to synthesize beta-galactosidase. Bacteria that have been transfected with pZ189 become ampicillin-resistant, and if the transfected plasmid carries a functioning *supF* gene, the bacteria will form blue colonies in agar containing 5-bromo-4-chloro-3-indolylβ-galactosidase (X-gal), and an inducer, isopropylβ-D-thiogalactoside. If the *supF* gene has been inactivated by a mutation, the colonies will be white or light blue. Plasmids isolated from these two kinds of colonies were analyzed for gross rearrangements by agarose gel electrophoresis. A portion of the plasmid DNA preparation was used to transform additional bacteria to ensure that the inability of the original transformants to utilize X-gal resulted from a mutant *supF* gene. The *supF* genes of the plasmids that again produced white or light blue colonies and exhibited normal patterns on gel electrophoresis were sequenced using dideoxynucleotides and ^{35}S-labeled adenosine 5'-[α-thio]triphosphate as described (Yang *et al.*, 1987). The polymerase used was modified T7 DNA polymerase (Sequenase) (United States Biochemical Corporation, Cleveland, OH).

(e) *Determination of sites AAF-DNA adducts*

To determine the frequency of adduct formation by N-AcO-AAF, we carried out polymerization reactions using the Klenow fragment of *E. coli* DNA polymerase I essentially as described by Yang *et al.* (1988). As designed by Moore and Strauss (1979), this assay measures the degree of inhibition of polymerization caused by the presence of a bulky distorting

DNA adducts or photoproducts. Two forms of labeling were employed: primer end-labeling with [γ-^{32}P]-labeled adenosine 5′-triphosphate (New England Nuclear, Boston, MA) and polymerization using ^{35}S-labeled adenosine-5′-[α-thio]triphosphate. End-labeling eliminates the need to take into account the number of adenine bases incorporated during polymerization, but the ^{35}S-label gives more distinct bands. Double-stranded plasmid containing 25 AAF adducts was denatured and annealed with the pBR322 *Eco*RI site primer as described (Yang *et al.*, 1988). This primer binds to the transcribed strand near the 5′ end of the *supF* gene, and polymerization copies the transcribed strand in a rightward direction. A leftward 20-mer primer was annealed to the position 211-230 of the nontranscribed strand to copy that strand. Since the length of DNA to be copied from either primer is ~ 220 nucleotides, the average number of adducts per strand of *supF* gene was approximately 0.5. With either method of labeling the newly synthesized DNA, DNA from the four dideoxy sequencing reactions, carried out on an untreated template, was electrophoresed on the same gel to serve as DNA size markers. To quantitate the frequency of chain termination, we measured the relative intensities of the bands on the autoradiograph of the gel containing end-labeled oligonucleotide fragments, using a digital image analyzer (Visage 110, Bio Image, Kodak, Rochester, NY) and whole band analysis software. This software provides band quantification by defining boundaries of bands based on inflection points and integrating the optical density of the whole band and presenting these data as a percent of the total density of the material being analyzed in each lane of the gel.

3. Results

(a) *Characterization of N-AcO-AAF-treated plasmids*

Figure 1A shows the number of dG-C8-AAF adducts (AAF residues) formed per molecule of plasmid as a function of the concentration of tritium-labeled N-AcO-AAF used. A linear relationship was observed. When the carcinogen-treated plasmids were assayed for ability to transform the bacterial cells to ampicillin resistance, the transforming ability was found to decrease in direct proportion to the number of AAF residues bound (Figure 1B). To lower the transforming activity of the treated plasmid to 37% of the untreated control, approximately 18 AAF residues per plasmid were necessary, a number significantly lower than the 36 dG-C8-AF adducts (AF residues) required to achieve the same effect in our earlier study with N-AcO-TFA-AF-treated plasmids (Mah *et al.*, 1989).

Figure 1C shows the frequency of *supF* mutants obtained when plasmids treated with various doses of N-AcO-AAF or untreated plasmids were transfected into human 293 cells and allowed to replicate, and the progeny plasmids were rescued and assayed for mutations in indicator bacteria. At lower doses, i.e., up to 20 AAF residues per plasmid, the frequency of mutants was very similar to what was seen with AF residues (Mah *et al.*, 1989). However, at higher level of adducts the data clearly indicate a non linear increase in mutant frequency. At 40 AAF residues per plasmid, the mutant frequency was two times higher than with AF residues, i.e., 45 X 10^{-4} and 20 X 10^{-4}, respectively. Plasmids carrying the highest number of AAF residues, i.e..70 adducts, gave a *supF* mutants frequency of 127.7 X 10^{-4}, which is 98 times higher than the background frequency of 1.3 x 10^{-4}.

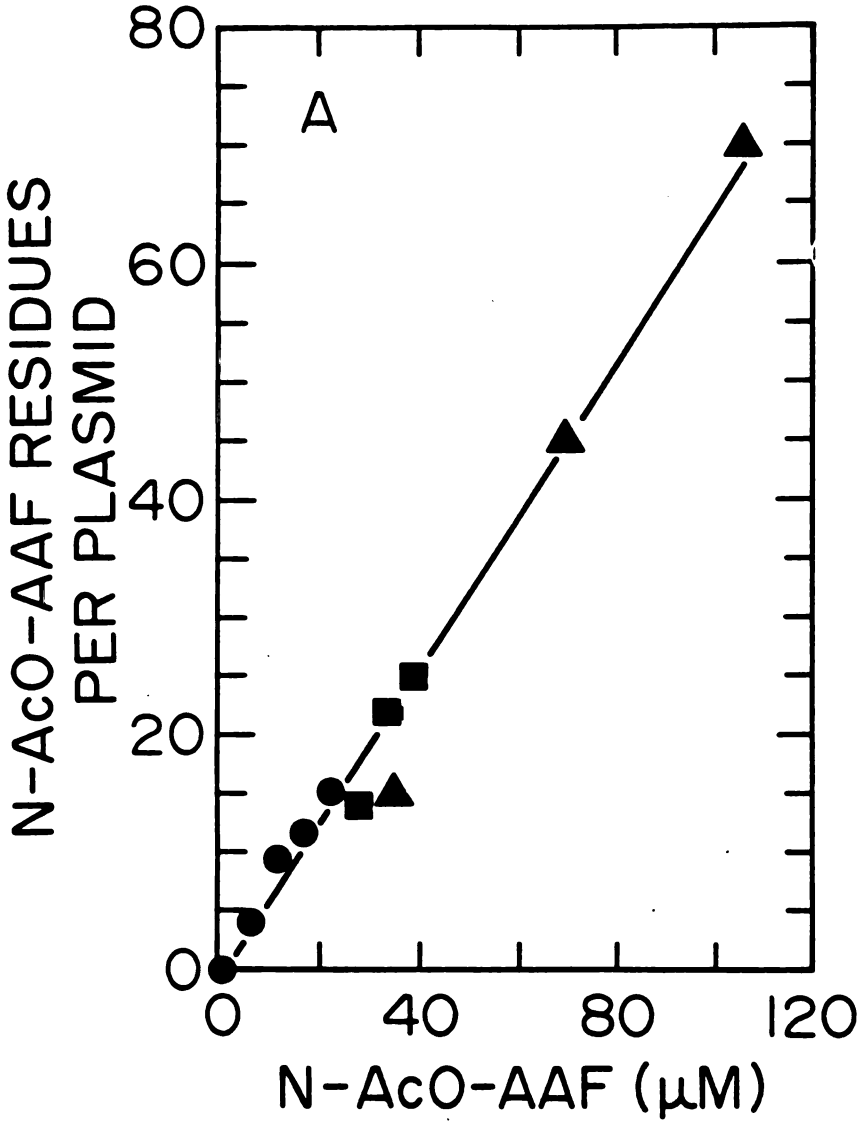

Figure 1. (A) Number of AAF adducts formed per plasmid as a function of concentration of radiolabeled N-AcO-AAF used in the *in vitro* reaction.

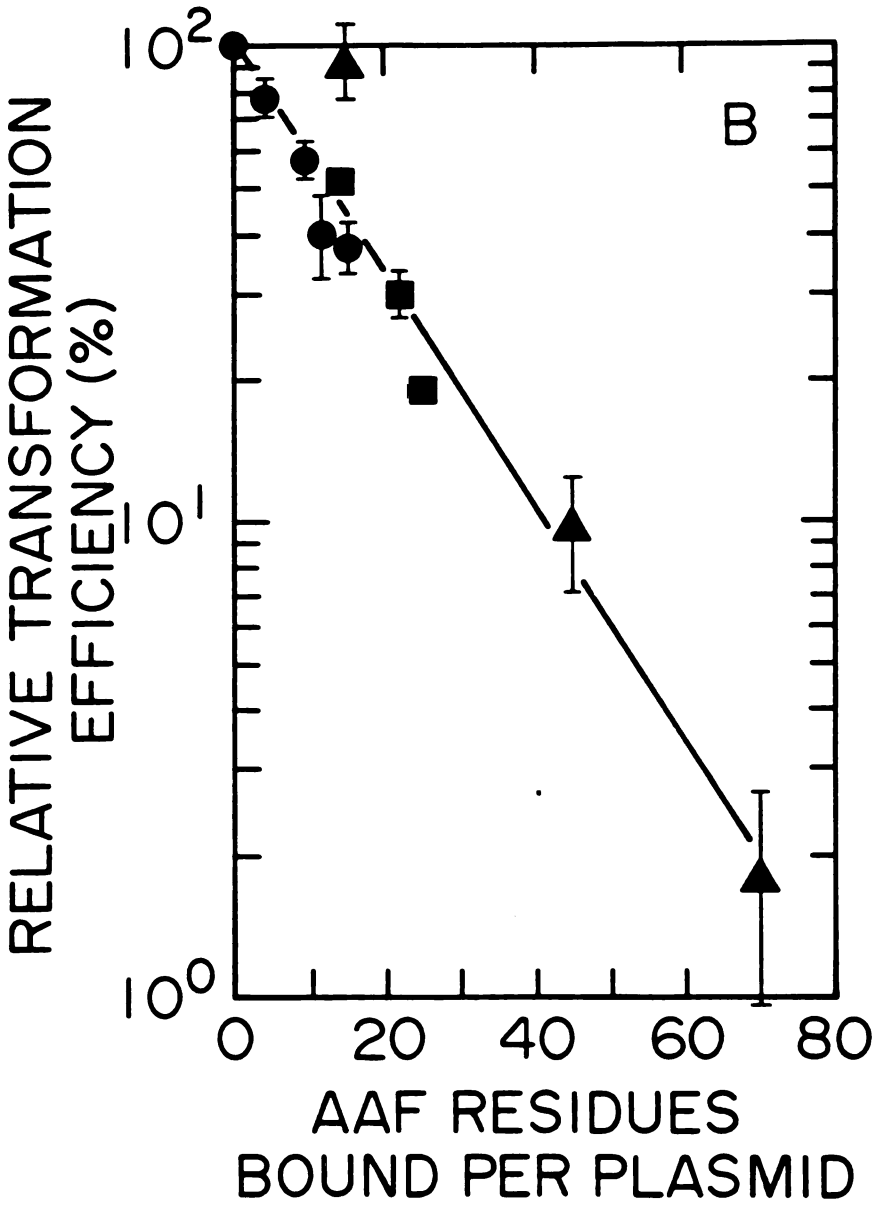

Figure 1. (B) Decrease in the ability of the plasmid to transform bacteria as a function of the number of AAF adducts per plasmid (the error bars indicate the standard errors of four determinations).

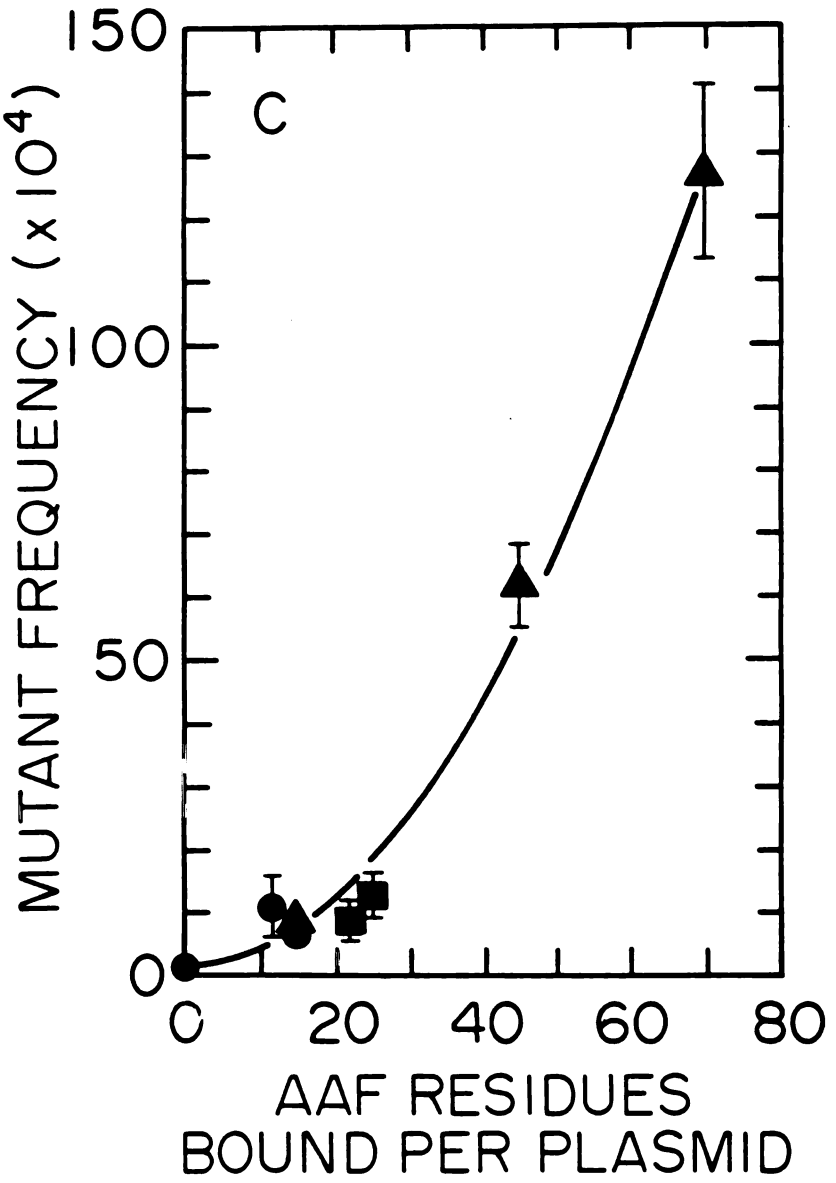

Figure 1. (C) Frequency of *supF* mutants induced when the plasmid replicated in the human host cells as a function of the number of AAF adducts per plasmid (the error bars refer to the standard errors of the *supF* mutant frequencies obtained from a series of individual human cell transfection experiments made with each set of treated plasmid).

Table I Spectrum Mutations by AAF Adducts

Analysis of mutants obtained by transformation of E. coli with progeny of N-AcO-AAF-treated pZ189 generated during replication in 293 cells

Adducts per plasmid	Number of human cell transfection experiments	*supF* mutants[a]/ transformants	Frequency of *supF* mutants (10^4)	Plasmids with altered gel mobility[b]/no. examined	Total plasmid *supF* genes sequenced	Characterization of sequenced mutants			Freq. of mutants with point mutations (10^4)[f]
						Number with deletions[c]	Number with insertions[d]	Number with point mutations[e]	
0	40	44/326744[g]	1.3	8/42	27	15	3	9	0.3
12	7	6/5552	10.8	0/6	4	0	0	4	10.8
15	13	33/48261	6.8	0/32	25[h]	1	0	23	6.3
15	3	9/10143	8.9	0/6	2	0	0	2	8.9
22	8	23/26487	8.7	0/22	17	1	0	16	8.2
25	8	16/12610	12.7	0/14	11	1	0	10	11.5
45	4	100/16068	62.2	0/28	16	0	0	16	62.2
70	4	89/6972	127.7	0/6	2	0	0	2	127.7

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

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

[c]Deletion of 9 to 150 bp.

[d]Insertion of 10 to 30 bp.

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

[f]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 9 divided by that in column 6 plus those mutants showing altered gel mobility (numerator, column 5).

[g]These data for the spontaneous mutants include previously published results obtained with untreated plasmids.

[h]One mutant with gross rearrangement.

As shown in the analysis of the N-AcO-AAF-induced *supF* mutants in terms of frequency, altered gel mobility, and type of mutations in Table I, the frequency of mutants containing point mutations in the *supF* gene in these plasmids exposed to the highest dose of N-AcO-AAF was 426 times higher than the frequency in the untreated plasmids.

A total of 42 mutant plasmids generated in human cells transfected with untreated control plasmids were analyzed for altered gel mobility to detect gross alterations (i.e., deletions or insertions of > 150 bp). Eight showed such alterations. In contrast, none of the 114 mutants obtained using N-AcO-AAF-treated plasmids that were analyzed showed detectable changes in gel mobility. Twenty-seven control mutant plasmids that did not show altered gel mobility, and 77 mutant plasmids obtained from N-AcO-AAF-treated plasmids were further analyzed by DNA sequencing of the *supF* gene to detect small deletions or insertions or point mutations, i.e., base substitutions or deletions or insertions of 1 or 2 bp. Eighteen of these 27 control background mutants (67%) contained deletions or insertions of 9 bp or more. In contrast, only 4 out of the 77 carcinogen- induced mutants analyzed (5%) showed alterations other than point mutations.

(b) *Spectra of mutations induced by N-AcO-AAF*

Table II shows the kinds of sequence alteration generated in the *supF* gene in both untreated and N-AcO-AAF-treated plasmids. This table includes only the unequivocally independent mutants (i.e., those in which either the mutation was unique or the plasmids were derived from independent cultures of transfected human cells). For purposes of

Table II

Comparison of sequence alterations generated in supF by replication of carcinogen-treated or untreated plasmid in human cells

Sequence alterations	Number of independent mutants sequenced					
	Control[a]		N-AcO-AAF		N-AcO-TFA-AF	
Single base substitution:	7		62		42	
Two base substitutions:						
Tandem	1	27%	0	85%	1	86%
≤ 20 bases apart	2		3		0	
> 20 bases apart	0		2		1	
Three base substitutions:						
≥ 20 bases apart	0		0		1	2%
Deletions:						
Single G•C pair	3		6		1	
Single A•T pair	1		0		0	
Tandem base pairs	0	51%	0	13%	0	8%
4-20 bp	7		3		2	
> 20 bases	8		1		1	
Insertions:						
Single G•C pair	1		1		0	
Single A•T pair	0	8%	0	1%	0	2%
≤ 20 bases	2		0		1	
Gross rearrangements:	5	14%	1	1%	1	2%
Total	37		79		51	

[a]These data for the spontaneous mutants include previously published results with untreated plasmids and recent results obtained with untreated pS189 plasmids which also contain the *supF* gene as the target gene for mutations.

comparison, the comparable data obtained previously for plasmids carrying AF residues, i.e., treated with N-AcO-TFA-AF (Mah *et al.*, 1989) are also included in Table II. The majority (85%) of the carcinogen-induced mutants exhibited one or two base pair substitutions. Of the spontaneous mutants, only 27% showed single or double base pair substitutions. Instead, 51% of the mutants from the untreated group contained deletions in the *supF* gene, and 79% of the deletions were greater than 3 base pairs. In contrast to N-AcO-TFA-AF, which only induced 1 single base pair deletion out of 51 mutants analyzed, approximately 8% (6/79) of the N-AcO-AAF-induced mutations consisted of single G•C base pair deletions.

The specific types of base substitutions found in the N-AcO-AAF modified plasmids, as well as in mutants obtained with untreated plasmids and those found in a previous study (Mah *et al.*, 1989) with N-AcO-TFA-AF-modified plasmids are listed in Table III for comparison. 97% (70/72) of the N-AcO-AAF induced base substitutions involved G•C pairs. Approximately 80% of mutations induced by both carcinogens were transversions, and 65% of those represented G•C --> T•A transversions. The majority of base substitutions found in the control mutants were also transversions (92%), with 69% being G•C --> T•A.

The specific locations of the point mutations derived from 74 N-AcO-AAF-induced independent mutants are shown in Figure 2. For convenience in making comparisons, the spectrum of mutations induced by N-AcO-TFA-AF (Mah *et al.*, 1989) is reproduced in this figure, as well as the spectrum of mutations observed in mutants from untreated control plasmids that replicated in the 293 cell line. The latter data include previously published results from our laboratory (Yang *et al.*, 1988; Mah *et al.*,

Table III

Comparison of the kinds of base substitutions generated in supF during replication of carcinogen-treated or untreated plasmid in human cells

Base change	Number of mutants observed		
	Control[a]	N-AcO-AAF	N-AcO-TFA-AF
Transversions:			
G•C --> T•A	9 (69%)	47 (65%)	32 (65%)
G•C --> C•G	1 (8%)	11 (15%)	8 (17%)
A•T --> T•A	0	0	0
A•T --> C•G	2 (15%)	0	0
Transitions:			
G•C --> A•T	1 (8%)	12 (17%)	9 (18%)
A•T --> G•C	0	2 (3%)	0
Total	13	72	49

[a]These data for the spontaneous mutants include previously published results with untreated plasmids and recent results obtained with untreated pS189 plasmids which also contain the *supF* gene as the target gene for mutations.

Figure 2. 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 rightward primer. The point mutations observed in the progeny of the treated plasmid are placed below the sequence. The mutations found in mutants from untreated plasmids are place above the line. The rectangle represents deleted nucleotide(s). The caret shows the location of an inserted cytosine. The bracket indicates that it is not possible to determine which nucleotide within a run is involved in the mutation. The mutations underlined represent tandem mutations. Every tenth base and the anticodon is underlined.

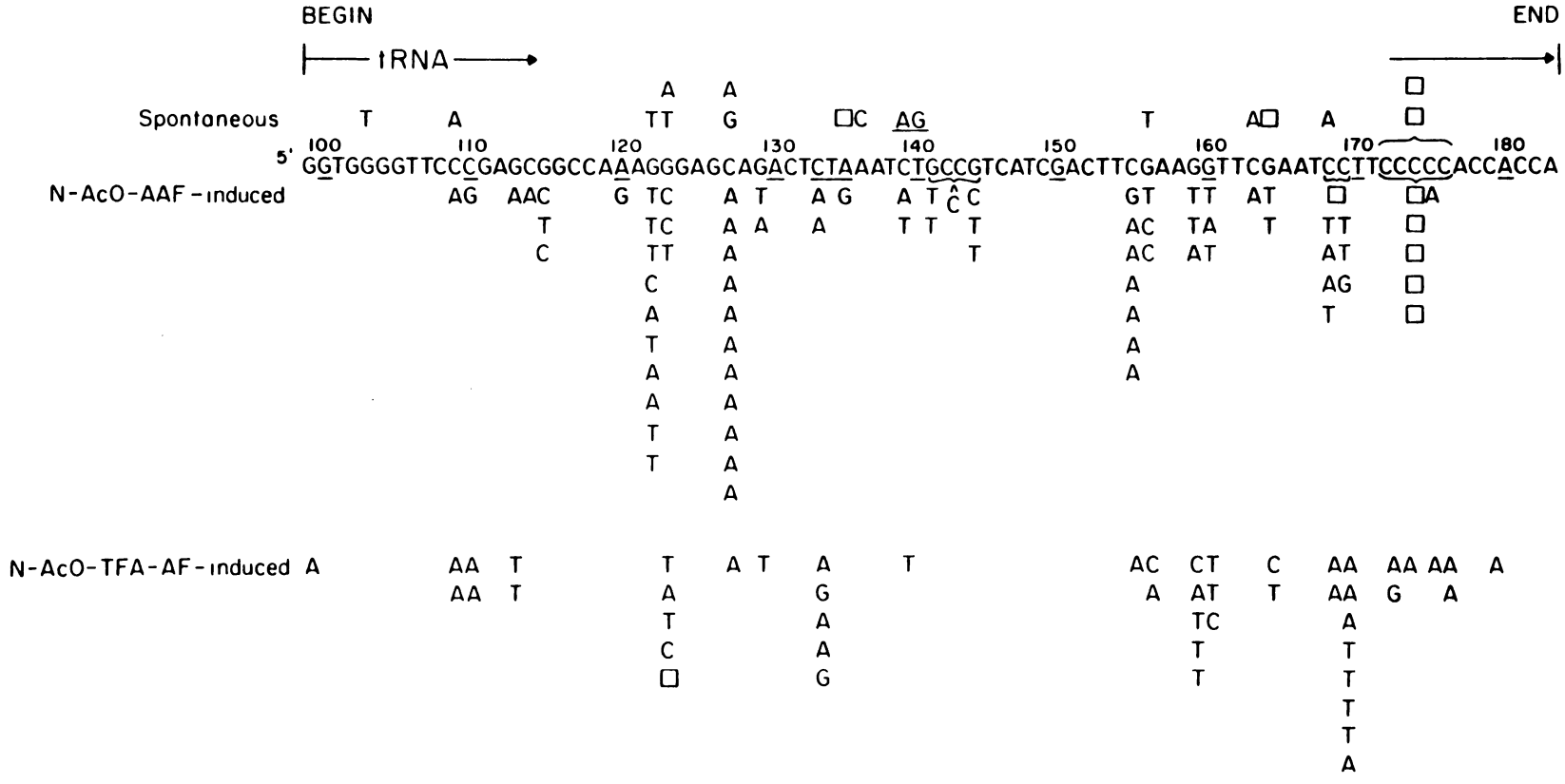
BEGIN
END
tRNA
Spontaneous
N-AcO-AAF-induced
N-AcO-TFA-AF-induced
5'
100
110
120
130
140
150
160
170
180
GGTGGGGTTCCCGAGCGGCCAAAGGGAGCAGACTCTAAATCTGCCGTCATCGACTTCGAAGGTTCGAATCCTTCCCCCACCACCA

Figure 2

1989, Boldt *et al.*, 1990). None of the "hot spots" were common to both carcinogens. Prominent "hot spots" for N-AcO-AAF-induced base pair substitutions were found at position 122, 127, 155, and a unique minus one frameshift mutation occurred frequently in the run of 5 G C base pairs found in region 172-176. In contrast, N-AcO-TFA-AF induced prominent base substitution "hot spots" at positions 123, 133, 159, and 169.

(c) Correlation between sites of AAF adducts and the location of AAF-induced mutations

To see if the "hot spots" for N-AcO-AAF-induced mutations reflected "hot spots" for DNA binding, the DNA polymerase termination assay of Moore & Strauss (1979) was carried out to determine the sites and frequencies of carcinogen-DNA adduct formation in the *supF* gene. This method of estimating the percentage of carcinogen adducts formed at particular sites on the gene relies on two assumptions: that the density of the bands in a sequencing gel is proportional to the number of DNA molecules of a particular length and that the length of the fragments reflects the chance of adduct-induced premature termination of polymerization. We used the *E. coli* DNA polymerase I (Klenow fragment) in our *in vitro* DNA synthesis assay since it has been shown not to bypass dG-C8-AAF adducts (Sahm *et al.*, 1989). Two primers, one rightward that anneals to the transcribed strand at the 5′ end of the *supF* gene and one leftward that anneals to the non-transcribed strand at the 3′ end of the gene, were used so that both the transcribed and the nontranscribed strands of the *supF* gene could be analyzed.

Figure 3 shows a diagram of the relative intensities of the

Figure 3. Relative frequency of carcinogen adducts in the 5′ to 3′ and 3′ to 5′ strand of the coding region of the *supF* gene, as judged by the polymerase-stop assay, and location of carcinogen-induced base substitutions. The Klenow fragment of DNA polymerase I was used with either the rightward or the leftward sequencing primer to determine polymerase-stop sites (panel shown above the coding sequence of the *supF* gene). The relative intensities of the bands on the autoradiograph were determined by digital image analyzer. The data, expressed as percent of the total band densities, are represented by the length of the lines. The base substitutions shown below the *supF* tRNA gene are the guanine base changes found in the corresponding strand.

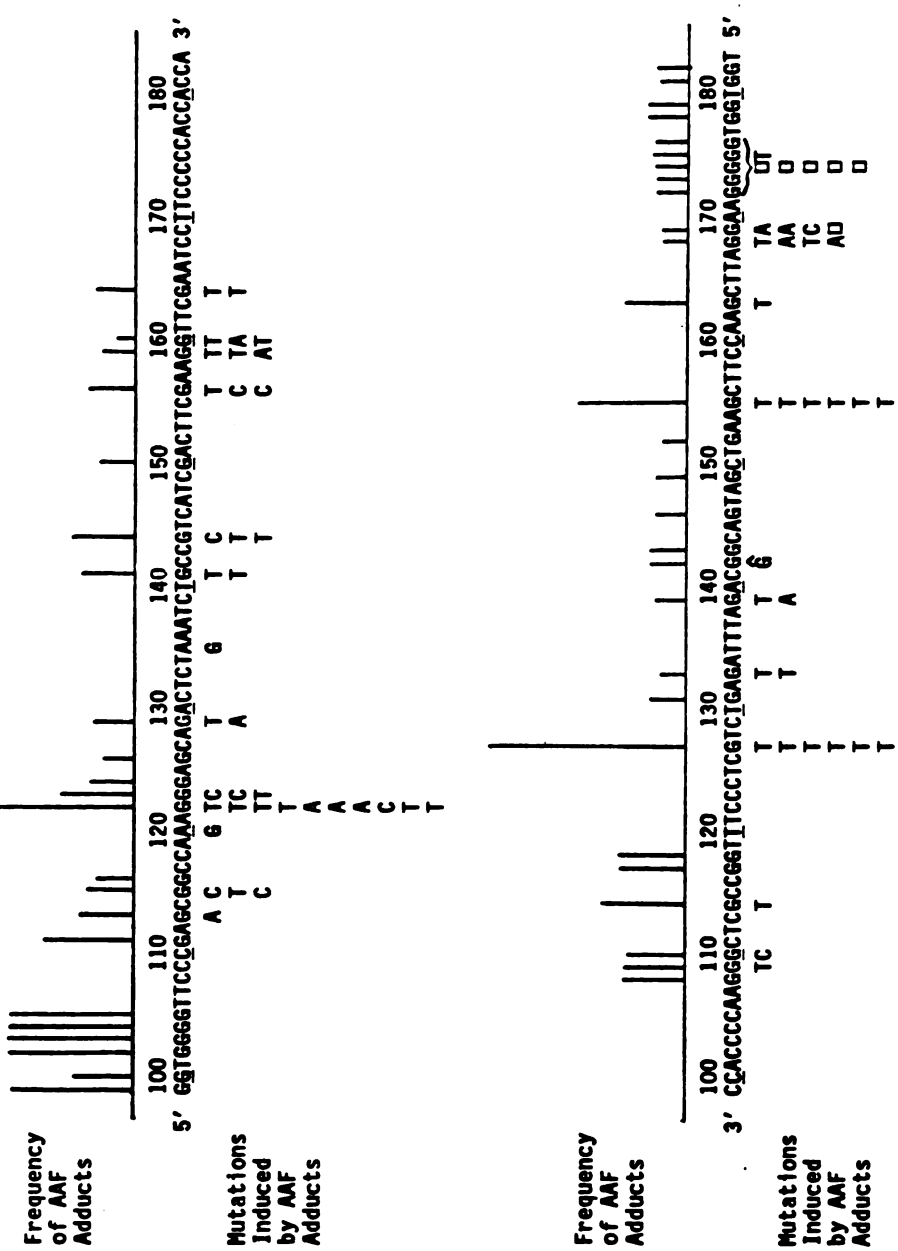
Frequency of AAF Adducts
100 110 120 130 140 150 160 170 180
5' GGTGGGTTCCGAGCGGCCAAAGGGAGCAGACTCTAAATCTGCCGTCATCGACTTCGAAGGTTCGAATCCTTCCCCCACCACCA 3'
Mutations Induced by AAF Adducts
Frequency of AAF Adducts
100 110 120 130 140 150 160 170 180
3' CCACCCCAAGGGCTCGCCGGTTCCCTCGTCTGAGATTTAGACGGCAGTAGCTTGAAGCTTCCAAGCTTAGGAAGGGGGTGGTGGT 5'
Mutations Induced by AAF Adducts

Figure 3

termination sites as judged from the intensities of the bands on the gel. Care was taken so that the autoradiographs of the polymerization termination gels had the right time of exposure, therefore offering samples of good quality for band density analysis. The pattern of the DNA bands obtained with DNA containing AAF adducts corresponded to positions one nucleotide prior to virtually every cytosine in the DNA-sequencing standard line, indicating that DNA synthesis was terminated one base prior to each guanine in the template. No bands corresponding to positions one nucleotide away from any base other than guanine were seen, and there was no evidence of any interference with polymerization when untreated templates were used. Each of the prominent sites for mutation induction (i.e., positions 122, 127 and 155) also showed a relatively high frequency of carcinogen binding, but some sites which showed high carcinogen-adduct formation were very "cold" for mutation induction (e.g., positions 99, 102-105, and 111).

An example of a polyacrylamide gel showing the sites of termination on a template containing 25 AAF adducts per plasmid (0.5 adduct per strand of the *supF* gene) is shown in Figure 4. In this particular assay the leftward sequencing primer was used to determine the location of adducts on the 5′ to 3′ strand of the *supF* gene (the non-transcribed coding strand), beginning at position 210. The arrow points to a band at position 123 that is notably darker than the others, indicating a "hot spot" for termination of polymerization. This corresponds to a termination site (adduct) at position 122 on the template which is a "hot spot" for AAF induced mutations. As noted in the Materials and Methods Section, both ^{35}S labeled adenosine - 5′- [α-thio] triphosphate and primers end-labeled

with [γ-^{32}P]-labeled adenosine 5′-triphosphate were used for the polymerase-stop assay. Both assays gave similar results. For quantitative analysis we used the sample with the ^{32}P end-labeled primer, since this eliminates the need to take into account the number of adenine base incorporated during polymerization. We used the ^{35}S-labeled sample for the photograph shown in Fig.4 because it gave sharper bands and could be more easily analyzed by eye.

Figure 4. Polyacrylamide gel showing the sites of termination in the *supF* gene of the Klenow fragment of *E. coli* polymerase I on a template containing 0.5 AAF adducts per 230 bases by using the *in vitro* DNA polymerase-stop assay of Moore and Strauss (1979), as described by Yang *et al.* (1988). The leftward sequencing primer was used to determine the location of adducts on the 5′ to 3′ strand. The lanes labeled GATC are dideoxy sequence standards obtained by incubating untreated DNA templates with sequenase and dideoxyribonucleotides. Lane 25 shows the product of the stop assay carried out on a template prepared from N-AcO-AAF treated pZ189 containing 25 AAF adducts per plasmid; lane 0 represents a template from untreated plasmid. The stop assay reactions for the templates shown here were run using ^{35}S-labeled adenosine 5′-[α-thio]triphosphate. The reaction was chased with unlabeled dATP for 15 min. The numbers on the left identify the location of bases in the *supF* gene. The arrow points to position 123.

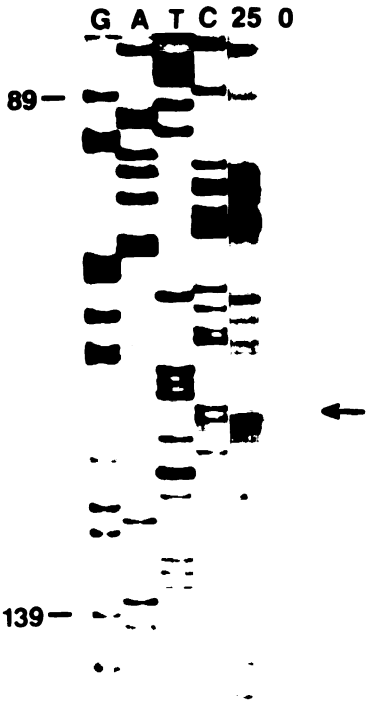

Figure 4

4. Discussion

The fact that 97% (70/72) of the base substitutions and 100% (7/7) of the single base pair insertions or deletions induced by AAF adducts occurred at G•C base pairs strongly suggests that the mutagenesis was targeted to sites where adducts occurred, since N-AcO-AAF forms adducts in DNA predominantly, if not, exclusively, with guanine (Johnson *et al.*, 1987). The base substitutions involving A•T pairs were observed in mutants derived with plasmids treated with lower doses of carcinogen and might reflect background mutation. Alternatively they might have resulted from a minor adduct involving AAF residues bound to adenine (Kapuler & Michelson, 1971; Kriek & Reitsema, 1971).

Support for this also comes from the results of the DNA polymerase-stop assay (Fig. 3). There was a good degree of correlation between mutation "hot spots" and the frequency of adduct formation at these sites. All three prominent sites for mutation induction (i.e., positions 122, 127 and 155) also showed a high frequency of carcinogen binding. This indicates that the spectrum of mutations is at least in part dependent on the preferential binding of the carcinogen to the specific sites. The fact that some sites showed high carcinogen-adduct formation, but very low mutation induction (e.g., positions 99, 102-105 and 111) can be explained by "silent mutations". Even though such sites have been shown to give a phenotype when they are mutated (Kraemer & Seidman, 1989), the kinds of mutations that have ever been scored at these sites do not include G•C -> T•A transversion, the major kind of mutation induced by dG-C8-AAF. For example, the only kind of mutation ever found at position 99 was an G•C -

-> A•T transition, whereas this type of mutation represents only 17% of the total of base substitutions induced by dG-C8-AAF in the *supF* gene. Both G•C --> A•T transition and G•C --> T•A transversion at position 111 have been found to cause a phenotype change in the *supF* gene. However, the latter transversion, which is the predominant kind of base substitution induced by dG-C8-AAF in this gene, was shown to give a phenotype only if it occurred as a tandem mutation involving this site. Similarly, even though G•C --> T•A transversions have been detected at positions 102-105 with other agents, dG-C8-AAF is probably not likely to induce this kind of mutation at this sequence. Koffel-Schwartz *et al.* (1984) showed that it induces mainly minus one frameshifts in a sequence constituted of repeated bases. Minus one base pair deletions have never been detected at positions 102-105 in the *supF* gene.

Various mechanisms could explain why 65% of the AAF- and AF-induced base substitutions were G•C --> T•A transversion. Such transversion mutations could result from apurinic sites in DNA when a polymerase inserts adenosine triphosphate into the opposite strand during replication (Kunkel, 1984; Loeb & Preston, 1986). However, dG-C8-AF and dG-C8-AAF have been shown to be stable adducts (Johnson *et al.*, 1987), making depurination unlikely to be an important event in the mutagenesis mechanism. Furthermore, Bichara & Fuchs (1985) have evaluated the mutagenic potency of the apurinic lesions in their assay and found that simple depurination events at adducted guanine residues resulting in G•C --> T•A transversion mutations would account for less than 10% of AF-induced mutations in their experiments. Another possibility is that DNA polymerases in the human cells preferentially insert an adenine nucleotide

opposite a noninstructional base containing a bulky adduct ("A" rule) (Strauss *et al.*, 1982). However, G•C --> C•G transversions and G•C --> A•T transitions were also found (each one representing ~ 17% of the base substitutions). If changes in the conformation of guanine caused by the AAF or AF residues allowed stable purine purine mispairing, which went undetected, this could explain the high proportion of G•C --> T•A transversions that are observed. Brown *et al.* (1986) showed that in a synthetic deoxydodecamer, unmodified guanine could pair stably with adenine in the *syn* position without distorting the helix. Using a different deoxydodecamer, crystal structures of G(*syn*) A(*anti*) mismatches could be observed at near-neutral pH (Brown *et al.*, 1989). Furthermore, Norman *et al.* (1989) found evidence that in a double-stranded oligonucleotide containing a single dG-C8-AF adduct, adenine opposite the modified guanine produced a stable structure which placed the AF in the B-DNA minor groove, with the guanine *syn*. Since AAF adducted guanine has been shown to assume the *syn* conformation in AAF modified DNA helix (Grunberger et al., 1970; Fuchs & Daune, 1972; Santella et al., 1980; Broyde & Hingerty, 1983), this might allow the replication machinery to insert an adenosine triphosphate opposite the modified guanine to form a stable mispairing. The G•C --> C•G and G•C --> A•T base changes could reflect less stable mispairing with guanine or thymine nucleotides opposite the modified guanine.

The AAF mutagenesis data in the present study involving human cells as the host for plasmid replication differ significantly from those obtained by Fuchs and co-workers (Fuchs *et al.*, 1981; Koffel-Schwartz *et al.*, 1984) when plasmids containing randomly distributed AAF adducts were

allowed to replicate in *E. coli*. Both studies made use of a forward mutation assay, i.e., loss of functional *supF* tRNA or loss of tetracycline resistance, respectively. In the studies by Fuchs and co-workers, >90% of the mutations induced were frameshifts. In ours, 85% were base substitutions. These differences in specificity between *E. coli* bacterial and human cells migth reflect the difference in target gene sequences. Alternatively, they might reflect a difference in the mechanism of mutagenesis between prokaryotes and eukaryotes. This is supported by fact that AAF and its metabolites, such as N-AcO-AAF, which form deacetylated dG-C8-AF adducts when administered to *Salmonella typhimuriun* bacteria, induce frameshifts (Ames *et al.*, 1972; Beranek *et al.*, 1982), whereas these same dG-C8-AF adducts induced mainly base substitutions in an endogenous gene in a Chinese hamster ovary (CHO) cell line, i.e., the dihydrofolate reductase gene of CHO cells (Carothers *et al.*, 1989). Cole and co-workers (1982) concluded from their studies in the mouse cell L5178Y that a structurally-related carcinogen, 1,8-dinitropyrene prefenrentially induced base substitutions, whereas in bacterial mutagenesis tests, 1,8-dinitropyrene was a strong inducer of frameshift mutations (Mermelstein *et al.*, 1981).

AAF residues were more efficient in causing mutations than AF residues, even though the kinds of mutations were very similar, i.e., both adducts induced mainly base substitutions (85%) in our assay. The former are more bulky adduct than the latter, and perhaps cause more interference with DNA replication. This also might explain why AAF residues induced a higher frequency of minus one base pair frameshift mutations, i.e., 8% compared to 2% induced by AF residues. Suggestion that

this is the case comes from the finding by Boldt *et al.* (1991) using the same assay that 1,6-DNP adducts are similarly more mutagenic than the less bulky 1-NP adducts. With 40 1,6-DNP adducts per plasmid the *sup F* mutant frequency was 37 x 10^{-4} compared to 23 x 10^{-4} for 1-NP. Like AAF adducts, 1,6-DNP also induced a significant proportion of minus one base pair frameshift mutations, i.e., 16% for 1,6-DNP compared to 1.5% for 1-NP.. The majority (>80%) of these frameshifts occurred in a run of 5 G's located at positions 172-176 in the non-coding strand of the *supF* gene. These single base pair deletions might be explained by the Streisinger strand slippage model (Streisinger *et al.*, 1986), as suggested by Koffel-Schwartz *et al.* (1984) in their study of N-AcO-AAF induced mutations in pBR322 plasmid replicating in *E. coli*.

It should be pointed out that a run of 3 G's (positions 122-124) and a run of 4 G's (positions 102-105) occur in the coding strand of the *supF* gene, as well as a run of 3 G's in the opposite strand (positions 108-110). The run of 4 G's is complementary to the run of 5 C's at position 172-176 within the same strand when the cloverleaf structure of the *supF* tRNA is formed. Yet >80% of the minus one frameshift mutations induced by either AAF or 1,6-DNP residues were located in the run of 5 G's. No frameshifts were found in the run of 3 G's or 4 G's. Although base substitutions have been found in the run of 4 G's (positions 102-105), no mutants containing a single base pair deletion in this region have ever been found in any of the numerous mutation studies using the *supF* gene (summarized by Kraemer & Seidman, 1989; see also Yang *et al.*, 1987; 1988; Bigger *et al.*, 1989; Mah *et al.*, 1989; Boldt *et al.*, 1991). The fact that no minus one frameshift mutations have ever been found in the run of 3 G's

(positions 108-110 and 122-124) can be explaned by the limited length of the repeated sequence. Similarly, Koffel-Schwartz *et al.* (1984) reported that in their assay, only in runs of four or five base pairs did they find a frameshift. In that same study there was a unique minus two base pair deletion (GC) which occurred at high frequency (50%) in their mutation assay using N-AcO-AAF-modified pBR322 plasmid replicated in *E. coli*. This kind of two base pair deletion was found in sequences that have been shown to be converted into Z-DNA by dG-C8-AAF, such as the sequence recognized by the restriction enzyme *Nar*I (5'GGCGCC-3'), or other alternating pyrimidine-purine sequences (Sage & Leng, 1980; 1981; Santella *et al.*, 1981; Wells *et al.*, 1982). Since the *supF* gene does not contain such sequences, their mutational specificity induced by dG-C8-AAF in an shuttle vector replicated in mammalian cells could not be investigated in the present study.

When the spectrum of mutations induced by N-AcO-AAF in the *supF* gene was compared to those previously found for other structurally related polycyclic carcinogens such as N-AcO-TFA-AF (Mah *et al.*, 1989), 1-NOP (Yang *et al.*, 1988), 1,6-DNP (Boldt *et al.*, 1991), BPDE (Yang *et al.*, 1987), and (-)B<u>c</u>PhDE-2 (Bigger *et al.*, 1989), some mutation "hot spots" were shared by two or more carcinogens. However, each carcinogen induced its own unique spectrum of mutations, probably by its unique mechanism. The fact that different sites of a gene with similar sequences (i.e., run of G's, G C sites) are affected in a different way by different, even though structurally-related carcinogens, indicates that factors other than primary structure, such as DNA-repair and DNA associated proteins (Kootstra, 1986), can also be important for determining susceptibility of

sites to mutagenesis.

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CHAPTER IV

Kinds of mutations found when a shuttle vector containing adducts of 1,6-dinitropyrene replicates in human cells

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SUMMARY

To gain insight into the mechanisms by which carcinogens induce mutations in human cells, we have been comparing the frequency and spectrum of mutations induced when a shuttle vector, pS189, carrying covalently-bound residues of structurally-related carcinogens, replicates in human 293 cells. In the present study, we investigated the mutagenic effects of *N*-hydroxy-1-amino-6-nitropyrene, a partially reduced derivative of 1,6-dinitropyrene (1,6-DNP). The results were compared with what was found previously in the same assay with *N*-hydroxy-1-aminopyrene, the partially-reduced derivative of 1-nitropyrene. The shuttle vector plasmids were exposed to tritiated 1-nitro-6-nitrosopyrene for 1 h in the presence of ascorbic acid, which served as a reducing agent to generate *N*-hydroxy-1-amino-6-nitropyrene. ^{32}P-postlabeling showed that only a single adduct was formed, i.e., *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene. There was a linear increase in the number of adducts per plasmid as a function of applied concentration and also in the frequency of *supF* mutants as a function of adducts per plasmid, reaching 58.8 x 10^{-4} above a background of 0.8 x 10^{-4}. When the frequency of mutants induced when plasmids carrying residues of 1,6-DNP replicated in the human cells was compared with that induced by 1-NP residues, the former was 1.8 times more mutagenic than the latter. Both carcinogens induced mainly base substitutions, primarily G·C --> T·A transversions; but 1,6-DNP adducts

produced a significant fraction of minus one frameshifts, with most of these located in a unique run of five G's in the gene. Polymerase termination reactions indicated that 1,6-DNP adducts were formed at every guanine, but not elsewhere in the gene. The "hot spots" for adduct formation were not perfectly correlated with "hot spots" for mutation induction. This indicates that the ultimate biological effect of the chemical depends not only on the number of adducts originally formed, but also on such processes as cellular DNA repair, which may remove such adducts from the plasmids before DNA replication occurs, as well as on the structure of the neighboring bases at the site of the adduct.

INTRODUCTION

Nitrated polycyclic aromatic hydrocarbons (nPAH) are common environmental contaminants produced primarily as the result of incomplete combustion processes (1 and reviewed in ref. 2). These compounds are found, for example, in urban air particulate (3), coal fly ash (4), and diesel exhaust emission (5, 6). The predominant nPAH in diesel exhaust is 1-nitropyrene (1-NP), but 1,3, 1,6-, and 1,8- dinitropyrene (DNP) have also been detected. These four compounds are potent mutagens in bacteria (7, 8) and in certain mammalian cell lines (9-11), but in the *Salmonella typhimurium* reversion assay, the dinitropyrenes are much more mutagenic than 1-NP. It has also been demonstrated that 1,3-,1,6-, and 1,8-DNP are tumorigenic in experimental animals (11-17). The compound 1-NP has also been reported to cause tumors, although not in all experiments (13, 15, 18-20).

Transformation of normal cells into tumorigenic cells is recognized to be a multistepped process, and at least one of the steps involved in the development of cancer involves mutations. Since 1,6-DNP has been shown to be one of the most mutagenic nPAH in certain bacteria and mammalian cell assays and strongly tumorigenic in experimental animals, it was of interest to investigate the mechanisms by which this carcinogen induces mutations. For this purpose we determined whether there are specific sites in a target gene at which the carcinogen preferentially causes mutations, and if so, whether these correspond to the preferential binding sites of the compound. It was also of interest to compare 1,6-

DNP with 1-NP in the same assay to see if there were significant differences between the two structurally-related carcinogens in the frequency and/or kinds of mutations they induce. This is because both carcinogens form DNA adducts primarily with guanine, binding covalently at the C-8 position (21-24).

Our assay system uses a shuttle vector that carries a defined target gene and is capable of replicating in mammalian cells as well as in bacteria (25, 26). This characteristic make it possible to isolate newly mutated genes and analyze them at the sequence level. The vector is treated with a chemical carcinogen *in vitro* and then transfected into human cells where the mutations occur. The plasmids are recovered from the human cells, and those containing a mutated target gene are identified in a bacterial system and amplified. The specific mutational changes and their location in the target sequence are determined by sequencing these mutants.

For our study of 1,6-DNP, we chose to use the shuttle vector pS189 (27), which is a deletion derivative of the pZ189 shuttle vector (25). The advantage of this vector is that it gives a lower background mutant frequency than the pZ189 vector (27). As the target DNA sequence, we used the *supF* gene, which codes for a tyrosine-suppressor tRNA and has been cloned in these two vectors. An advantage of this target gene is that its small size makes it easy to analyze at the sequence level and ideal for determining "hot spots" and "cold spots" for mutation induction by carcinogens, i.e., sites where there is a non-random distribution of mutations. A further advantage of using the *supF* gene as the target gene is the high sensitivity of the tRNA to base changes. This sensitivity is

such that single base pair changes at almost any site in the structural region of the tRNA can lead to a detectable phenotypic change (28). As the eucaryotic host for plasmid replication, we used the human kidney cell line 293 (29), which gives a low spontaneous background frequency of *supF* mutants (30).

Because 1,6-DNP requires a two-step metabolic activation process before it can react with cellular macromolecules, it was not possible to treat the plasmid DNA with the parent compound. Instead, we made use of a partially-reduced metabolic intermediate, *N*-hydroxy-1-amino-6-nitropyrene, which forms the same DNA-adduct as the parent compound (23). The results of our study indicated that, as was the case when the corresponding partially-reduced metabolite of 1-NP, i.e., *N*-hydroxy-1-aminopyrene, was used in this assay system (24), there was a linear relationship between the number of 1,6-DNP adducts per plasmid and the frequency of *supF* mutants induced. The frequency of mutants induced per 1,6-DNP DNA-adduct was 1.8 times higher than for 1-NP adducts. Most of the induced mutations were base substitutions or single base deletions, of which 97% involved G-C base pairs, predominantly G·C --> T·A transversions. Even though both compounds exhibited several "hot spots" in common for mutation induction, each had its own unique site of preferential mutation induction on the gene.

MATERIALS AND METHODS

Cells and plasmids. The human embryonic kidney cell line, 293, which served as the eukaryotic host for the shuttle vector, as well as the ampicillin sensitive indicator bacteria host (SY204) carrying an amber mutation in the chromosomal -galactosidase gene, have been described (30). The 5337 bp shuttle vector, pS189, was constructed by Seidman (27) and contains the tyrosine amber suppressor tRNA gene (*supF*) flanked by two genes essential for its recovery in bacteria under the selective conditions.

Formation of 1,6-DNP adducts on the plasmid. [4,5,9,10-^{3}H]1-nitro-6-nitrosopyrene (764 mCi/mmol) was synthesized from [4,5,9,10-^{3}H] 1,6-DNP (Chemsyn Science Laboratories, Lenexa, KS) as described by Andrews *et al.* (31). Plasmids were prepared by using an alkaline lysis procedure (32) and purified by ethidium bromide - CsCl density gradient centrifugation. DNA dissolved in 10 mM sodium citrate buffer, pH 5.0, at 300-500 g/ml was added to a freshly prepared ethanol solution of 1-nitro-6-nitrosopyrene and incubated at 37°C for 2 h in the presence of ascorbic acid, which served as a reducing agent to generate *N*-hydroxy-1-amino-6-nitropyrene (23). Unbound compound was removed by phenol-chloroform extractions and three successive ethanol precipitations. The DNA was dissolved in 10 mM Tris, 1 mM EDTA pH 8.0 and stored at -20°C until used. The number of residues bound per mole of plasmid was calculated from the A_{260} absorption profile of the DNA and the specific activity of the carcinogen, using 1 OD = 50 μg DNA/ml.

^{32}P-postlabeling of adducts. ^{32}P-postlabeling of DNA adducts was performed with 0.2 μg pS189 DNA diluted with 2 μg carrier DNA using slight modifications of the n-butanol enhancement procedure of Gupta (33). DNA adducts were then separated by the contact-transfer method of Lu *et al.* (34). A 70% fraction of the ^{32}P-labeled DNA digest was applied 1.5 cm from the bottom of a 10 x 20 cm PEI-cellulose sheet (Machery-Nagel). Development in the first direction (D1) was with 0.65 M sodium phosphate, pH 6.0. Solvents used for adduct separation in D3 and D4 (on 10 x 10 cm Merck PEI-cellulose plates) were 3.6 M lithium formate, 8.5 M urea, pH 3.5, and 1.2 M lithium chloride, 0.5 M Tris-HCl, 8.0 M urea, pH 8.0, respectively. An additional development in 0.9 M sodium phosphate, pH 6.8, was performed in the direction of D4 onto a 3 cm Whatman No. 1 wick. Adducts were located by autoradiography, as described (35).

Transfection of human host cells and rescue of replicated plasmid. The procedures used to introduce the plasmids into the 293 cells and rescue the progeny plasmids were essentially as described (30). Briefly, the cells were plated into a series of 100-mm diameter dishes at a density of 2 - 3 x 10^6 cells/dish, incubated under growth conditions for 24 h, and transfected with 5 μg of treated or untreated plasmid per dish using calcium phosphate co-precipitation. The cells were allowed to grow for an additional 48 h before low- molecular-weight DNA (plasmid) was extracted and purified. Plasmids harvested from individual dishes were kept separate from each other so that mutations occurring frequently ("hot spots") and putative siblings arising from within the same population could be distinguished. Prior to bacterial transformation, the plasmids were treated with *Dpn*I to digest any DNA that had the methylation pattern

of the bacteria used to prepare the original input plasmids.

Bacterial transformation and characterization of mutant supF genes. The techniques used were essentially as described (30). Briefly, progeny plasmids were assayed for mutant *supF* genes by transforming SY204 bacterial cells to ampicillin resistance and plating them on Luria-Bertani agar containing 5-bromo-4-chloro-3-indolyl β-D-galactosidase (X-gal), isopropyl β-D-thiogalactoside, and ampicillin. Cells containing plasmids with an inactive *supF* form light blue or white colonies, rather than dark blue colonies, because the tRNA is unable to suppress the amber mutation in the bacterial -galactosidase gene. Mutant colonies were restreaked on these agar plates to confirm the phenotype, and the bacterial plasmids were amplified and extracted, analyzed by agarose gel electrophoresis, and a portion of the DNA was used to transform SY204 bacteria once more to ensure that the inability of the original transformants to utilize X-gal was the result of an inactivated *supF* gene. Mutants that showed no change in gel electrophoretic pattern were amplified and purified for DNA sequence analysis using either CsCl/ethidium bromide centrifugation (30) or a small scale alkaline lysis procedure essentially as described by Zagursky *et al.* (36). Purified *supF* gene was sequenced as described (30), using the dideoxyribonucleotide method and ^{35}S-labeled adenosine 5′-[α-thio]triphosphate and the modified T7 DNA polymerase (Sequenase, United States Biochemical) following the manufacturer's recommended protocol.

Determination of sites of carcinogen-induced adducts. An estimation of the positions of 1,6-DNP adducts in the *supF* gene of carcinogen-treated plasmids was made using the in vitro DNA polymerase-stop assay of Moore and Strauss (37) essentially as described previously

(24), except that the reactions were run not only with ^{35}S labeled adenosine-5′-[α-thio]-triphosphate as in reference 24, but also with the primer end-labeled with [$\gamma^{32}P$]-labeled adenosine 5′-triphosphate (New England Nuclear, Boston, MA). Although the ^{32}P procedure gives somewhat less distinct bands, it is especially useful because it eliminates the need to take into account the number of adenine bases incorporated during polymerization. Briefly, double-stranded plasmids with a 1,6-DNP adduct level of 37 adducts per plasmid (i.e., 0.6 adducts of *supF* gene per strand) were denatured and annealed with the appropriate primer. Two 20-mer primers were used. One was complementary to the transcribed strand at position -29 to -10 relative to the beginning of the 200 bp *supF* gene; the other was complementary to the nontranscribed strand at position 211-230 relative to the *supF* gene. The polymerization reaction was performed in a manner similar to the sequencing reaction, except that the dideoxynucleotides were omitted, and the 1,6-DNP adducts were allowed to cause the termination. DNA from the four dideoxy sequencing reactions, carried out on an untreated template, was electrophoresed on the same gel to serve as DNA size markers. The relative intensities of the bands on the autoradiograph of the gel using [γ-^{32}P]-labeled adenosine 5′-triphosphate end-labeled primer were determined by a digital image analyzer (Visage 110, Bio Image, Kodak, Rochester, NY) using the whole band analysis software. This software provides band quantification by defining boundaries of bands based on inflection points and integrating the optical density of the whole band and presenting these data as percent of the total density of the material being analyzed in each lane of the gel.

RESULTS

Characterization of 1,6-DNP-treated plasmids. To determine the frequency of mutants induced by 1,6-DNP adducts and the nature of the mutations in the *supF* gene, plasmid pS189 was treated with various concentrations of tritium-labeled 1-nitro-6-nitrosopyrene in the presence of ascorbic acid, which served as a reducing agent to generate *N*-hydroxy-1-amino-6-nitropyrene. Treatment of DNA with *N*-hydroxy-1-amino-6-nitropyrene under slightly acidic conditions results in the formation of one major adduct, *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene (dG-C8-1-A-6-NP) (23). Likewise, only one adduct was detected when treated plasmids were analyzed by ^{32}P-postlabeling (Figure 1). Plasmids with various carcinogen adduct levels up to 44 adducts per plasmid and untreated plasmids were introduced into human cells and allowed to replicate. After 48 h, the progeny plasmids were harvested and introduced into the indicator bacteria *E. coli* SY204 in order to identify those carrying mutations in the *supF* gene.

The number of residues bound per plasmid and their ability to interfere with transformation of bacterial cells to ampicillin resistance was determined (30). As shown in Figure 2A, there was a linear increase in the number of 1,6-DNP adducts per plasmid as a function of the concentration of carcinogen used, except for the plasmids exposed to 35 M 1-nitro-6-nitrosopyrene. This batch of plasmids exhibited an adduct per plasmid level of only 38.5, instead of the 53 predicted by linear extrapolation. The high degree of interference of such adducts with the ability of the DNA to transform bacteria to ampicillin resistance (Figure

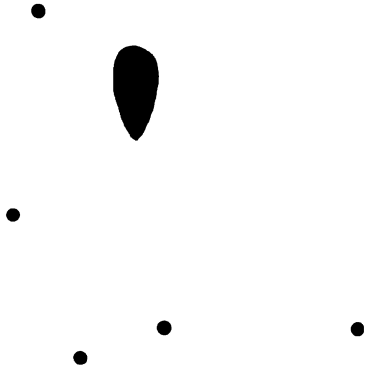

Figure 1. Autoradiogram obtained from ^{32}P-postlabeling pS189 DNA that had been reacted with *N*-hydroxy-1-amino-6-nitropyrene. Previous results (23) have shown that this treatment results in the formation of one major adduct, *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene.

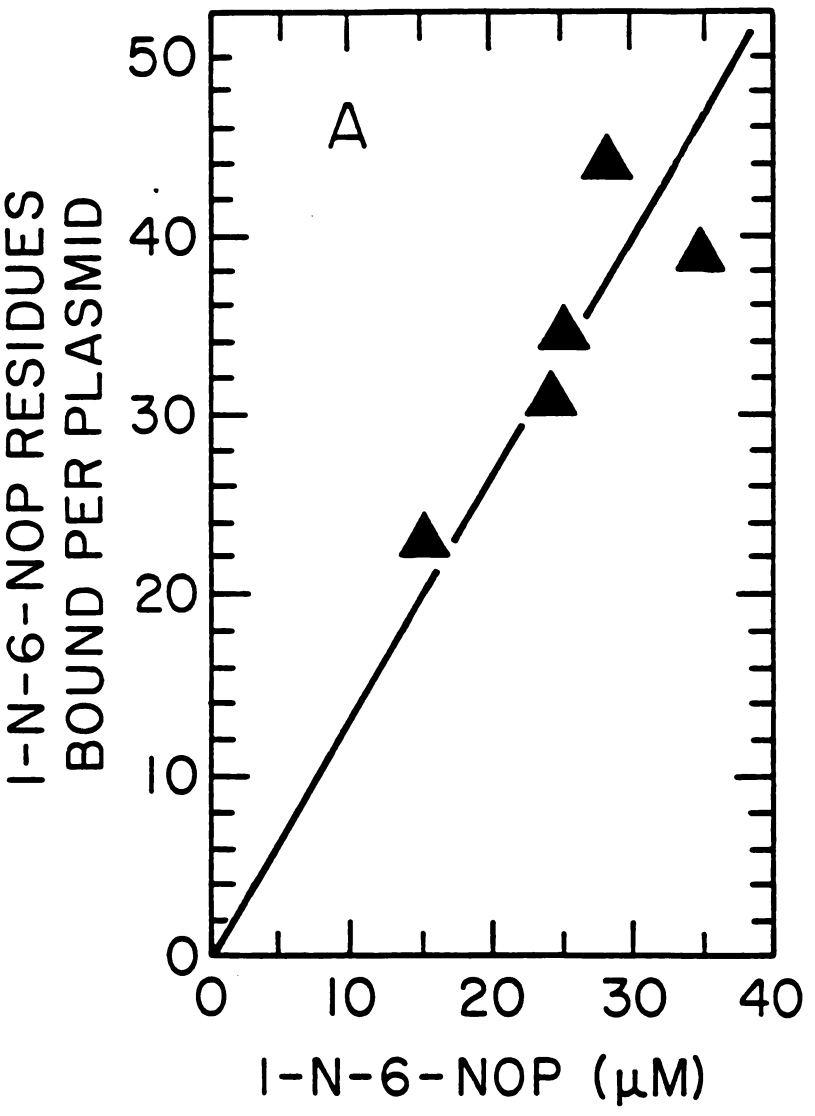

Figure 2. (A) Number of adducts formed on plasmid DNA as a function of concentration of labeled carcinogen used in an *in vitro* reaction.

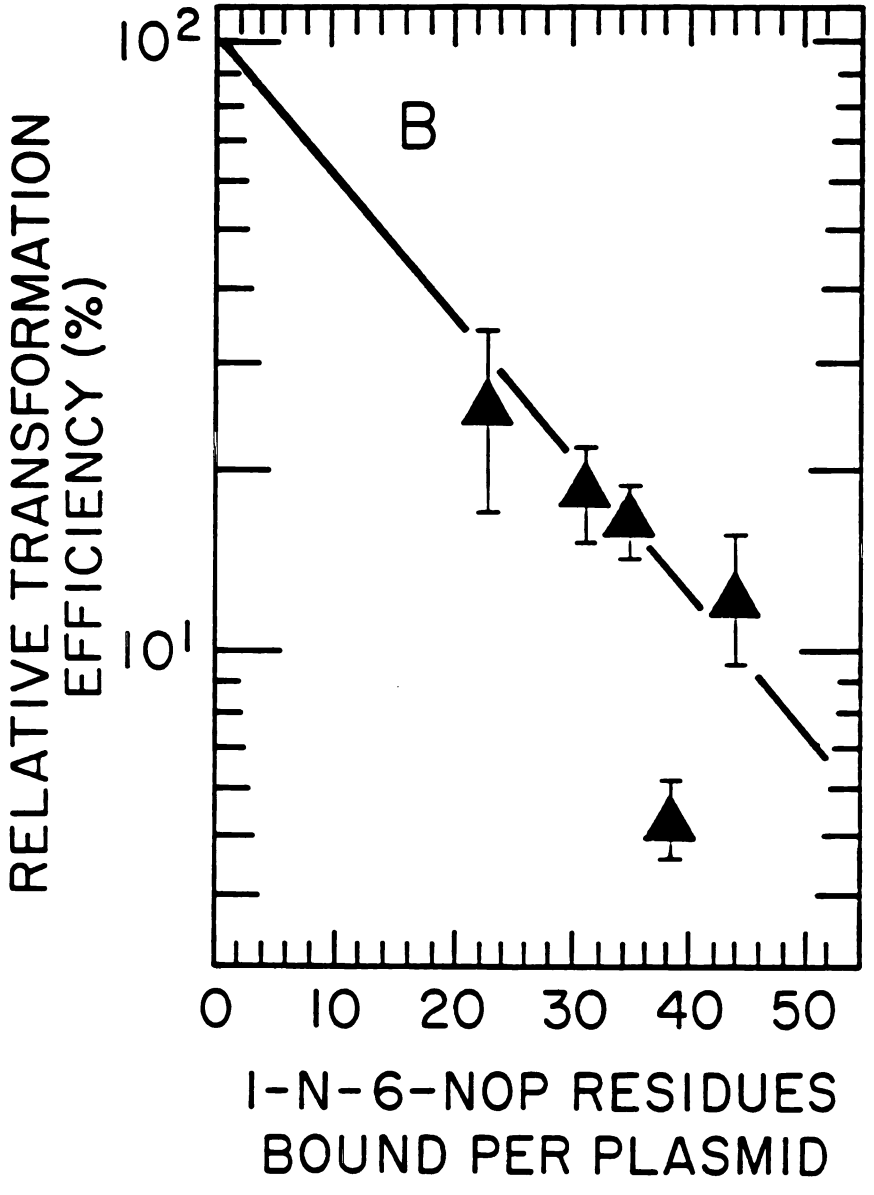

Figure 2. (B) Decrease in the ability of the plasmid to transform bacteria as a function of the number of adducts per plasmid (the error bars indicate the standard errors of four determinations).

2B) shown by that particular batch of plasmids, (i.e., 5% of the control), as well as the high mutation frequency found with this particular batch of plasmids (59 X 10^{-4}) (Figure 2C) supports the suggestion that the mean number of adducts per plasmid actually was closer to 53 than 38.5. Approximately 19 1,6-DNP residues bound per plasmid were necessary to lower the transforming activity of the modified plasmid to 37% of the untreated control plasmids. In contrast approximately 7 1-NP residues bound per plasmid lower the transforming activity of the modified plasmid to the same level. The highest mutant frequency induced by 1-6-DNP was 58.8 X 10^{-4}, which is 72 times higher than the background frequency of 0.8 X 10^{-4}. Compared with the frequency of mutants induced by 1-NP, 1,6-DNP was 1.8 times more mutagenic than 1-NP.

A total of 124 mutants from 1,6-DNP-modified plasmids and 32 from untreated plasmids were assayed in agarose for altered electrophoretic mobility to detect alterations (i.e., deletions or insertions > 150 bp). As expected for pS189, only 3 out of 124 induced mutants and 3 out of 32 control mutants showed an altered mobility in 0.8%agarose (Table I). The plasmids were retested by a second transformation study in SY204 cells to be certain that the mutation was in the *supF* gene and not a bacterial mutation, and then 123 mutants from 1,6-DNP-modified plasmids and 20 from untreated plasmids were analyzed by DNA sequencing (Table I).

Kinds of mutations induced by 1,6-DNP. The kinds of mutations induced in the unequivocally-independent mutants (i.e., those in which the mutation was unique or those derived from independent human cultures) are shown in Table II. For purpose of comparison, the comparable data for *N*-hydroxy-1-amino-pyrene obtained previously (24) are included in Table II.

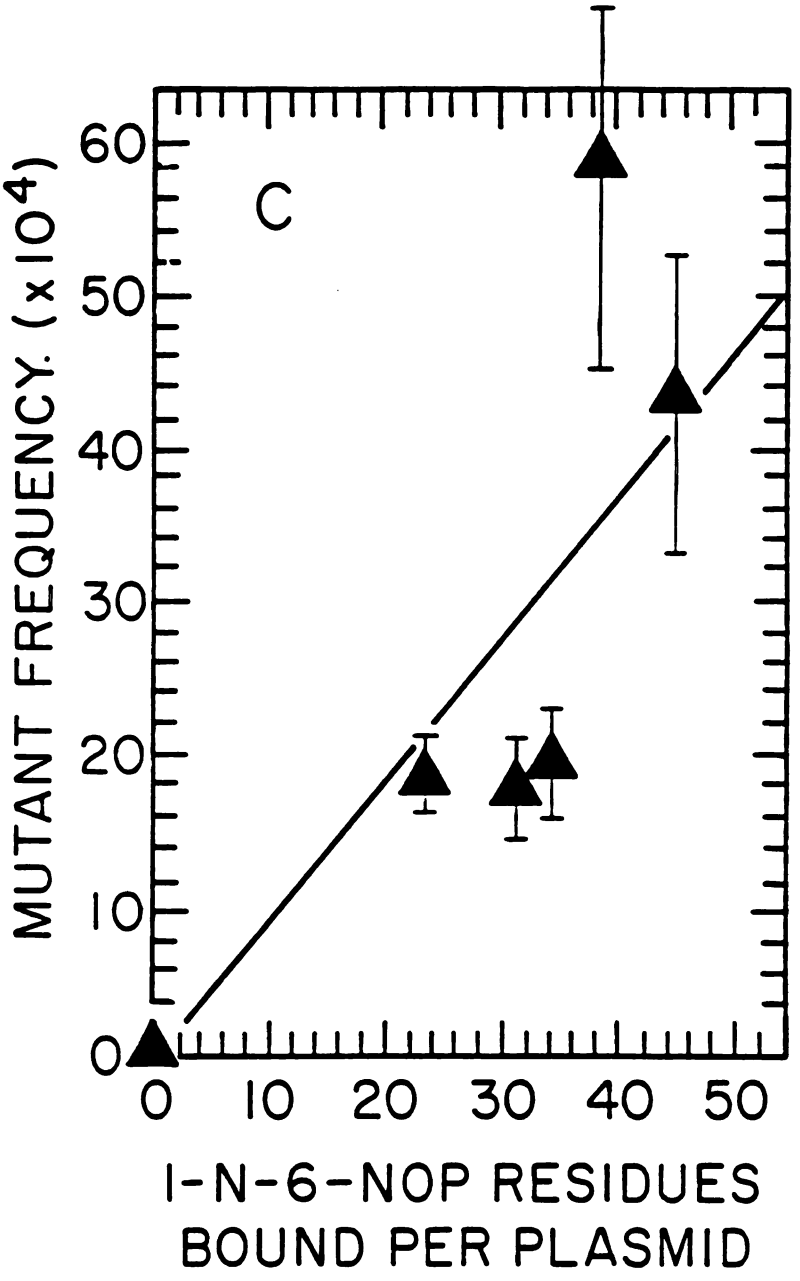

Figure 2. (C) Frequency of *supF* mutants induced when the plasmid replicated in the human host cells (the error bars refer to the standard errors of the *supF* mutant frequencies obtained from a series of individual human cell transfection experiments made with each set of treated plasmid).

Table I. Analysis of mutants obtained by transformation of *E. coli* with progeny of 1,6-DNP-modified pS189 generated during replication in 293 cells

Adducts per plasmid	Number of human cell transfection experiments	*supF* mutants[a]/ transformants	Frequency of *supF* mutants (10^{-4})	Plasmids with altered gel mobility[b]/no. examined	Total plasmid *supF* genes sequenced	Characterization of sequenced mutants			Freq. of mutants with point mutations (10^{-4})[f]
						Number with deletions[c]	Number with insertions[d]	Number with point mutations[e]	
0	13	32/390781	0.8×10^{-4}	3/32	15	4	0	6	0.3
23	6	42/22606	18.6×10^{-4}	1/39	38	1	1	36	17.2
31	5	24/13830	17.4×10^{-4}	0/24	24	0	0	24	17.4
34	1	26/13426	19.4×10^{-4}	1/26	26	0	1	25	18.0
38	1	17/2893	58.8×10^{-4}	0/17	17	0	0	16	55.3
44	5	18/4163	43.2×10^{-4}	1/18	18	0	0	17	38.7

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

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

[c]Deletion of 9 to 150 bp.

[d]Insertion of 10 to 30 bp.

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

[f]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 9 divided by that in column 6 plus those mutants showing altered gel mobility (numerator, column 5).

Table II. Comparison of sequence alterations generated in *supF* by replication of carcinogen-treated plasmid in human cells

Sequence alterations	Number of independent mutants sequenced					
	Control[a]		1,6-DNP-modified		1-NP modified[b]	
Single base substitution:	7		65[c]		48	
Two base substitutions:						
Tandem	1	27%	5	74%	2	85%
≤ 20 bases apart	2		2		0	
> 20 bases apart	0		1		1	
Deletions:						
Single G•C pair	3		16		1	
Single A•T pair	1		0		0	
Tandem base pairs	0	51%	0	19%	0	10%
4-20 bp	7		2		3	
21-100 bases	8		1		2	
Insertions:						
Single G•C pair	1		0		0	
Single A•T pair	0	8%	0	2%	1	5%
< 20 bases	2		0		2	
21-100 bases	0		2		0	
Tandem deletion/substitution:	0		4[c]	4%	0	
Gross rearrangement	5	14%	1	1%	0	
Total	37		98		60	

[a]These data for the control mutants include our previously published results with the *supF* gene (24, 39).
[b]These data are taken from ref. 24 with permission.
[c]One mutant has both one base substitution and one tandem deletion/substitution.

The majority, 74%, of the 1,6-DNP-induced mutants contained one, or at most two, base substitutions. This is similar to what was found with 1-NP adducts. However, in contrast to 1-NP adducts, which mainly produced base substitutions, 1-6-DNP adducts induced a significantly high frequency of single base pair deletions, i.e., 16 out of 98 independent mutants analyzed contained a single base pair deletion compared to 1 out of 60 for 1-NP adducts. In contrast to these induced mutations, the majority of the mutations in the control plasmids were deletions or insertions larger than 4 bp (46%) or gross rearrangements (14%). Only 27% of the mutations in the control plasmids were base substitutions.

Table III lists the kinds of base substitutions found in the 1,6-DNP-modified plasmids and compares them to the control plasmids and what was found previously for 1-NP (24). As expected from the adduct characterization data in Figure 1, the majority (97%) involved G·C base pairs.

The specific locations of the 1-6-DNP-induced mutations and the controls are shown in Figure 3. For convenience in making comparisons, the spectrum of mutations induced by 1-NP adducts (24) is reproduced in this figure. In analyzing our data, we define a "prominent hot spot" as any site at which at least 8% of the point mutations in a sample size of at least 50 observed point mutations, were located. We define a "less prominent hot spot" as any site where at least 5% of such mutations were located. Three prominent "hot spots" were found (positions 123, 144, 159). In addition, the region 172-176 represented a "hot spot" for minus one frameshift mutations. Since this region consists of a run of five G·C base pairs, one cannot assign the specific location of the deletion. Four

Table III. Comparison of the kinds of base substitutions generated in *supF* during replication of carcinogen-treated plasmid in human cells

Base substitution	Number of mutants observed		
	Control[a]	1,6-DNP modified	1-NP modified[b]
Transversions:			
G•C --> T•A	9 (69%)	52 (64%)	33 (61%)
G•C --> C•G	1 (8%)	10 (15%)	8 (15%)
A•T --> T•A	0	1 (1%)	3 (5.5%)
A•T --> C•G	2 (15%)	0	1 (2%)
Transitions:			
G•C --> A•T	1 (8%)	15 (18%)	6 (11%)
A•T --> G•C	0	2 (2%)	3 (5.5%)
Total	13	81	54

[a]These data for the control mutants include our previously published results with the *supF* gene (24, 39).
[b]These data are taken from ref. 24 with permission.

Figure 3. 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 rightward primer. The point mutations observed in the progeny of the treated plasmid are placed below the sequence. The mutations found in mutants from untreated plasmids are place above the line. The rectangle represents deleted nucleotide(s). The caret shows the location of an inserted thymidine. The star and the bracket indicate that it is not possible to determine which nucleotide within a run is involved in the mutation. The mutations underlined represent tandem mutations. Every tenth base and the anticodon is underlined.


BEGIN
tRNA
END
Spontaneous
1-N-6-NOP-induced
1-NOP-induced
5' GGTGGGGTTCCCGAGCGGCCAAAGGGAGCAGACTCTAAATCTGCCGTCATCGACTTCGAAGGTTCGAATCCTTCCCCCACCACCA
100
110
120
130
140
150
160
170
180

Figure 3

less prominent hot spots were found (positions 109, 127, 156, and 168). Two of the prominent "hot spots" for 1,6-DNP (positions 123 and 159) and three of the less prominent spots (109, 127, and 156) were the same for 1-NP, but the prominent hot spot at position 144, as well as the less prominent hot spot at position 168, were unique for 1,6-DNP adducts. In addition, only 1,6-DNP induced minus one frameshifts in region 172-176.

Correlation between sites of binding by 1,6-DNP and sites of mutations. To determine whether these mutational "hot spots" might correspond to "hot spots" for carcinogen binding in the *supF* gene, we carried out the DNA synthesis-stop assay of Moore and Strauss (37). This assay is based on the ability of bulky adducts to interfere with DNA replication. The mean number of adducts in the 1,6-DNP-modified plasmids that were analyzed in this stop assay was 37 per 5337 bp, i.e., 0.6 adducts per strand of the *supF* gene, if one assumes a random distribution. The transcribed strand, as well as the nontranscribed strand of the *supF* gene, was analyzed. With the carcinogen-treated sample, the bands corresponded to positions one nucleotide 5′ to virtually every cytosine in the DNA sequencing standard lane, which indicated that DNA synthesis was terminated one base prior to each guanine in the template. In one run of three G′s, the polymerization also terminated directly opposite the last G. No bands corresponding to positions one nucleotide away from any base other than guanine were seen, and there was little or no interference with polymerization on the untreated control template. As noted in Materials and Methods, for the polymerase-stop assay ^{35}S labeled adenosine-5′-[a-thio]triphosphate, as well as ^{32}P, end-labeled primer were used. Both assays gave similar results. For qualitative analysis, we used the

^{35}S-labeled samples because it gave sharper bands (Figure 4). For quantitative analysis we used the ^{32}P end-labeled samples (Figure 5).

Figure 5 diagrams the relative frequency of the polymerase terminations in both strands of the *supF* gene and shows the location of the corresponding mutations. The very prominent sites for mutation induction at position 144 and in the region 172-176, as well as the less prominent sites at positions 109 and 127, also exhibited a high frequency of chain termination (adduct formation). However, there was relatively little interference with polymerization at position 159, even though this was a very prominent mutational "hot spot". Some, but not all, of the lack of correlation between adduct formation and mutation frequency can be explained by so-called "silent mutations". For example, position 142 and 146 exhibited a high frequency of chain termination but no mutations (for 142) and only one tandem mutation (for 146). Cumulative data from studies with the *supF* gene (24, 28, 30, 38, 39 and unpublished studies) demonstrate that no mutations have ever been found at position 142, and that mutations at position 146 cause a phenotypic change only if there is a corresponding mutation at another site.

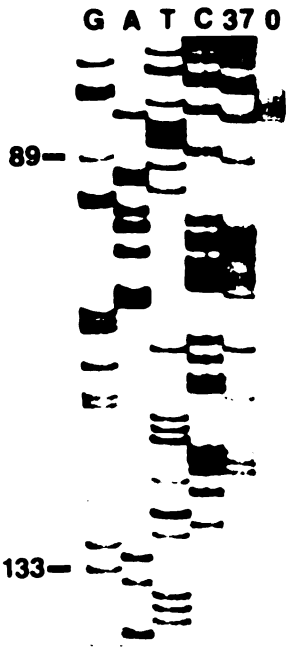

Figure 4. Polyacrylamide gel showing the sites of termination in the *supF* gene of the Klenow fragment of *E. coli* polymerase I on a template containing 0.6 1,6-DNP adducts per 230 bases by using the *in vitro* DNA polymerase-stop assay of Moore and Strauss (37), as described in reference (24). The lanes labeled GATC are dideoxy sequence standards obtained by incubating untreated DNA templates with sequenase and dideoxyribonucleotides. Lane 37 shows the product of the stop assay carried out on a template prepared for 1-nitro-6-nitrosopyrene treated pS189 containing 37 1,6-DNP adducts per plasmid; lane 0 represents a template from untreated plasmid. The stop assay reactions for the templates shown here were run using ^{35}S-labeled adenosine 5′-[α-thio]triphosphate. The reaction was chased with unlabeled dATP for 15 min. The numbers on the left identify the location of bases in the *supF* gene.

Figure 5. Relative frequency of carcinogen adducts in the 5′ to 3′ and 3′ to 5′ strand of the coding region of the *supF* gene, as judged by the polymerase-stop assay, and location of carcinogen-induced base substitutions. The Klenow fragment of DNA polymerase I was used with either the rightward or the leftward sequencing primer to determine polymerase-stop sites (panel shown above the coding sequence of the *supF* gene). The relative intensities of the bands on the autoradiograph were determined by digital image analyzer. The data, expressed as percent of the total band densities, are represented by the length of the lines. The base substitutions shown below the *supF* tRNA gene are the guanine base changes found in the corresponding strand.

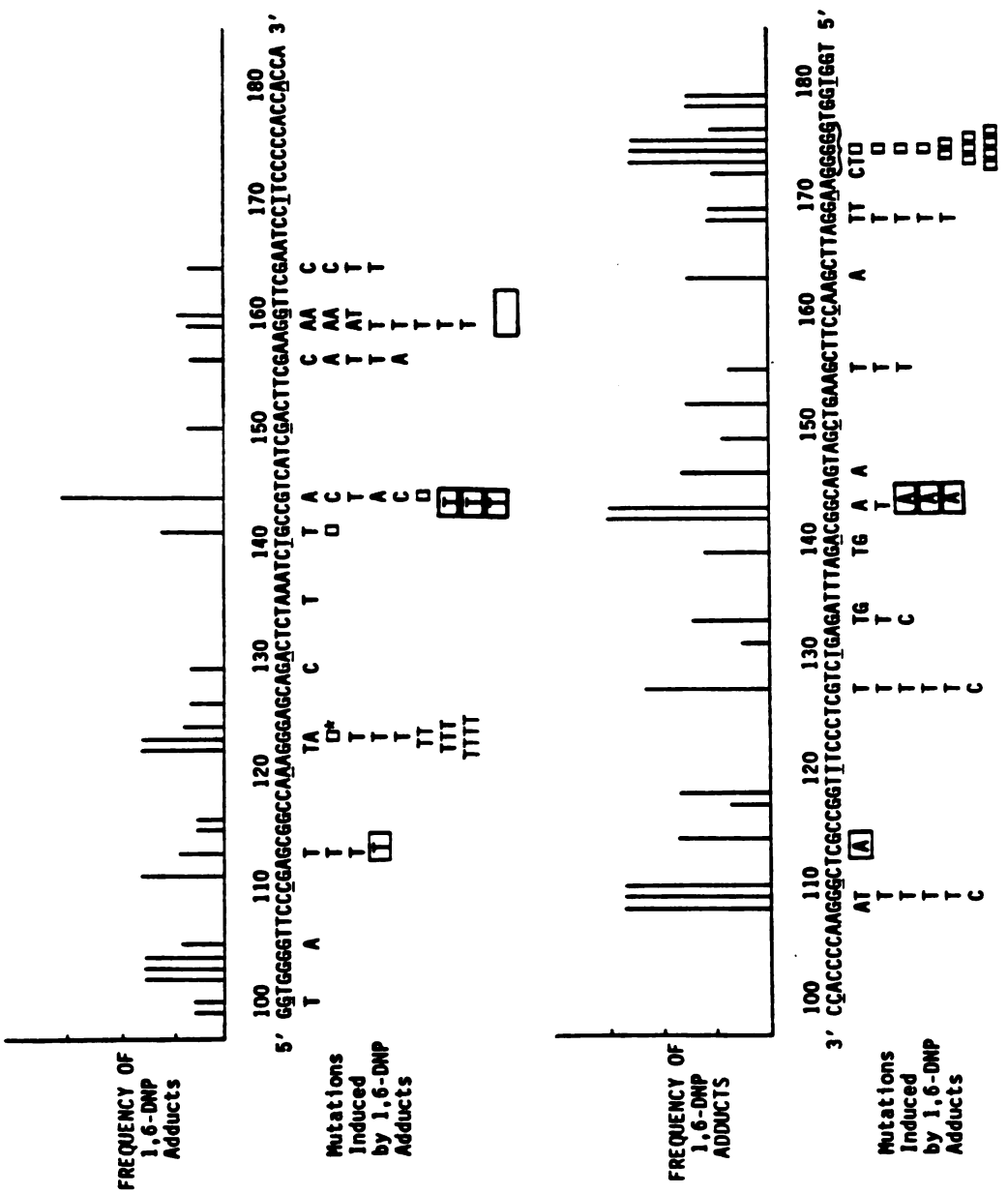
FREQUENCY OF 1,6-DNP Adducts
5' GGTGGGGTTCCCGAGCGGCCAAGGGAGCAGACTCTAAATCTGCCGTCATCGACTTCGAAGGTTCGAATCCTTCCCCCACCACCA 3'
Mutations Induced by 1,6-DNP Adducts
FREQUENCY OF 1,6-DNP ADDUCTS
3' CCACCCCAAGGGCTCGCCGGTTTCCCTCGTCTGAGATTTAGACGGCAGTAGCTGAAGCTTCCAAGCTTAGGAAGGGGTGGTGGT 5'
Mutations Induced by 1,6-DNP Adducts

Figure 5

DISCUSSION

The present study was undertaken to determine the frequency and specific kinds of mutations induced when pS189 carrying DNA adducts formed by 1,6-DNP replicates in human cells and to compare them with the results that have been found in the same system using the structurally-related carcinogen 1-NP. An advantage of the plasmid mutagenesis system is that one can compare the mutagenic activity of structurally-related carcinogens on the basis of equal numbers of adducts per plasmid. The frequency of adduct formation by 1-nitro-6-nitroso-pyrene in the presence of ascorbic acid was two fold lower than with an equal concentration of 1-nitrosopyrene (cf. Figure 2A with Figure 1A of ref. 24). Perhaps *N*-hydroxy-1-amino-6-nitropyrene is more readily reduced to the amino (i.e., 1-amino-6-nitropyrene), or perhaps the compounds have slightly different reactivities. What is noteworthy is that while 1,6-DNP adducts caused less interference with bacterial cell transformation than did 1-NP adducts, they were almost twice as efficient as 1-NP adducts in causing mutations. For example, 20 1,6-DNP adducts per plasmid gave 19 mutants per 10^4 plasmids whereas the same number of 1-NP adducts gave 10. Nevertheless, the majority of mutations induced by either carcinogen were single base substitutions, primarily G•C --> T•A transversions.

These results are in accordance with the prediction of Cole *et al.* (40) who concluded from their studies in the mouse cell L5178Y that 1,8-DNP, a compound very closely related to 1,6-DNP, also preferentially induced base substitutions. In contrast to this, in bacterial mutagenesis tests, DNPs are strong inducers of frameshift mutations (41). These

differences in specificity between bacterial and mammalian cells imply a significant difference in mechanisms of mutagenesis between those cells.

The fact that the majority of base substitutions induced by 1,6-DNP adducts, just as by 1-NP adducts, occurred mainly at G•C base pairs strongly suggests that the mutagenesis by 1,6-DNP is targeted to sites where adducts occur since *N*-hydroxy-1-amino-6-nitropyrene binds predominantly, if not exclusively, to guanine (Figure 1). A very small fraction (3%) of the base substitutions involved A•T base pairs. 17% of the background base substitutions involved A•T base pairs, but it is unlikely that the A•T base substitutions we observed with the 1,6-DNP-modified plasmids represented background mutations since the frequency of mutants was always at least 20-fold higher than background.

Several mechanisms could explain why 64% of the 1,6-DNP induced base substitutions were G•C --> T•A transversions. Changes in the conformation of guanine by the adduct might allow stable purine•purine mispairing (42, 43). Norman *et al.* (43) presented indirect evidence that in a oligonucleotide, a guanine with a structurally-related polycyclic aromatic carcinogen covalently bound to the C8 position was able to form a stable base pair with adenosine triphosphate. The guanine was rotated to the *syn* position. Brown *et al.* (42) showed that in a synthetic deoxydodecamer, guanine could pair stably with adenine in the *syn* position without distorting the helix. If stable base pairing occurred between dG-C8-1-A-6-NP and adenine in our assay, and the mismatch was not always recognized in the human cells (42), this would give the observed transversion. Another possibility is that the human cell polymerases preferentially insert adenine opposite a non-instructional lesion, viz., dG-C8-1-A-6-NP

(44) or opposite an apurinic site (45). Less frequent insertion of a C or a T opposite a non-instructional dG-C8l-A-6-NP (45) might explain why these kinds of base substitutions were so infrequent.

1,6-DNP adducts induced a high frequency of minus one frameshift mutations. Most of these frameshift mutations occurred in a run of 5 G's at a specific site on the gene (position 172-176). The mechanism by which this particular kind of mutation was caused might be the Streisinger strand slippage model (46). It is difficult to explain why no such mutations were found in the run of 4 G's in the opposite strand of the plasmid (positions 102-105), even though binding occurred there (Figure 5). This might be the result of preferential repair of adducts from particular regions. Another explanation could be that the long run of G•C's base pairs is more likely to allow slippage than the shorter run. It is also difficult to explain why 1,6-DNP adducts induced such minus one frameshifts, but 1-NP adducts did not. The former adducts are larger and therefore, perhaps, more bulky than the latter, and this may have played a role in the induction of minus one frameshifts since we have recently observed that acetylaminofluorene guanine adducts cause such mutations in this same system (Mah *et al.*, unpublished studies), whereas aminoflourene guanine adducts do not (39).

Unlike the spontaneous mutations, the induced mutations were not randomly distributed in the *supF* DNA sequence, but occurred preferentially at certain sites. 1,6-DNP exhibited three prominent "hot spots", two in common with 1-NP. The other two were unique for 1,6-DNP. The fact that the kinds and location of mutations caused by 1,6-DNP and 1-NP were not identical indicates that both carcinogens, despite the fact that they are

structurally closely related cause their own spectrum of mutations by unique mechanisms. Two of the hot spots for mutations induced by 1,6-DNP were located in a run of G's. One of them, in the region 172-176, which is a hot spot for a minus one frameshift mutation, is unique for 1,6-DNP and not found in the spectrum of 1-NP or for other structurally-related polycyclic aromatic carcinogens, i.e., (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE) (30), *N*-acetoxy-*N*-trifluoroacetyl-2-aminofluorene (N-AcO-TFA-AF) (39) and benzo[*c*]phenanthrene(4*R*,3*S*)-dihydrodiol (2*S*,1*R*)-epoxide (38). Since the mutations found at this site occurred in a run of 5 G's, one cannot know which of the bases was deleted. The other hot spot were at the middle G in a run of 3 G's (position 123), a hot spot also for mutations induced by 1-NP, BPDE, as well as N-AcO-TFA-AF (24, 30, 39), but not for N-AcO-AAF (Mah *et al.*, unpublished studies). None of the sites with G's in a row were sensitive to mutation induction by (-)-BcPhDE-2 (38). There are two other sites in the *supF* gene with three G's or four G's in a row (108-110) and (102-105), respectively. The former was sensitive for mutations induced by 1,6-DNP adducts; but the latter was not.

The fourth prominent hot spot for 1,6-DNP adducts, located at position 159 which might be part of a hairpin structure, is also a hot spot for 1-NP adducts. The fact that different sites of a gene with similar sequences (i.e., run of G's, G·C sites) are affected in different ways by 1,6-DNP and 1-NP indicates that factors other than primary structure, such as DNA-repair or DNA associated proteins (47), are important for determining susceptibility of sites to mutagenesis.

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CHAPTER V

SUMMARY AND CONCLUSIONS

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To get insight into the mechanisms by which carcinogens cause mutations in human cells, I studied and compared the spectra of mutations induced by four structurally-related carcinogen adducts during shuttle vector replication in human cells. All four adducts are formed at the C-8 position of guanine. dG-C8-AF and dG-C8-AAF adducts result from reaction of metabolic derivatives of the liver carcinogen 2-acetylaminofluorene with DNA. dG-C8-1-NP and dG-C8-1,6-DNP adducts are the results of environmental pollutants present in the diesel exhaust, 1-nitrosopyrene and 1-nitro-6-nitrosopyrene.

The results of the research presented in Chapters II, III and IV, combined with previous findings from our laboratory showed that :

1. The mutations observed in our studies were targeted to the adducts formed because (a) the frequency of mutants correlated with the increase of the dose and the number of adducts per plasmid; (b) the kinds of mutations found in carcinogen-treated plasmid were different from those found in control untreated plasmids; (c) the point mutations involved almost exclusively base pairs to which the carcinogen specifically binds; (d) no mutations were observed at sites where termination of polymerization by the Klenow fragment was not detected; (e) there was a good correlation between the sites of carcinogen binding and sites of

2. When compared on the basis of the number of adducts per plasmid, AAF and 1,6-DNP residues exhibited similar mutagenic potency which was about two times higher than that of AF and 1-NP residues.

3. All four residues induced mainly base substitutions (80%). However, AAF and 1,6-DNP residues showed a significantly higher frequency of minus one base pair deletions compared to AF and 1-NP residues. Since AAF and 1,6-DNP residues are more bulky than AF and 1-NP residues, they may cause more interference with DNA replication. This could explain why the former are more mutagenic than the latter. It could also expalain the higher frequency of minus one frameshifts as the result of slippage during DNA replication.

4. About 65% of the base substitutions were G • C - - > T • A transversions. This can be explained if changes in the conformation of guanine caused by the carcinogen adducts allowed stable purine•purine mispairing which went undetected through replication. Data from other investigators support this theory. The G•C--> C•G and G•C--> A•T base changes could reflect less stable mispairing with guanine or thymine nucleotides opposite the modified guanine.

5. The mutations induced by the different carcinogens used were not randomly distributed and the locations of "hot spots" and "cold spots" for mutations were unique for each agent.

6. There was good correlation between the sites of carcinogen binding as determined by the DNA polymerase stop assay and the sites of mutations, indicating that at least in part the occurrence of mutational "hot spots" and "cold spots" reflects the preferential binding of the

carcinogen to certain sites.

7. Excision of DNA adducts from particular sites in the target gene before replication occurs may also play a role in determining the mutation spectrum.

The results from the present work, together with the results of the investigations of others, support the following conclusions :

1. The results obtained with studies using AF, AAF and dinitropyrenes conducted in both systems inply that there is a significant difference in molecular mechanisms of mutagenesis between bacteria and mammalian cells.

2. The similarity of results obtained from the studies of the specific kinds of mutations induced by AF and BPDE adducts in both shuttle vector and mammalian endogenous gene systems show that shuttle vectors are reliable useful tools in the studies of mutagenesis in mammalian cells.