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Kinds and spectra of mutations formed when a shuttle vector containing adducts of benzo(a)pyrene-7,8-diol-9,10-epoxide or 1-nitrosopyrene replicates in mammalian cells

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KINDS AND SPECTRA OF MUTATIONS FORMED WHEN A SHUTTLE VECTOR CONTAINING ADDUCTS OF BENZO[a]PYRENE-7,8-DIOL-9,10-EPOXIDE OR 1-NITROSOPYRENE REPLICATES IN MAMMALIAN CELLS

by

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ABSTRACT

KINDS AND SPECTRA OF MUTATIONS FORMED WHEN A SHUTTLE VECTOR CONTAINING ADDUCTS OF BENZO[a]PYRENE-7,8-DIOL-9,10-EPOXIDE OR 1-NITROSOPYRENE REPLICATES IN MAMMALIAN CELLS

by

Jia-Ling Yang

Polycyclic aromatic hydrocarbons such as benzo[a]pyrene (BP) and nitrated derivatives such as 1-nitropyrene (1-NP) are widespread environmental contaminants produced by incomplete combustion. The principal reactive metabolite of BP is the anti isomer of (±)BP-7,8-diol-9,10-epoxide (BPDE) which binds to DNA mainly at the N^2 position of guanine. The reactive metabolite of 1-NP, 1-nitrosopyrene (1-NOP) is further reduced and binds covalently to DNA at the C8 position of guanine. Since the kinds of tumors formed by the two parent carcinogens differ and since the DNA adducts differ, they may cause mutations by different mechanisms.

To examine this question, a shuttle vector (p3AC), carrying a bacterial suppressor tRNA target gene (supF), was treated with BPDE. The BPDE-treated plasmid was introduced into a monkey cell line (COS7) and allowed to replicate. Progeny plasmids were then isolated,

purified, and introduced into bacteria carrying an amber mutation in the β-galactosidase gene to detect those carrying mutations in the supF gene. The background frequency of supF mutants obtained was too high to detect BPDE-induced mutants, but electrophoretic analysis showed that mutants derived from treated plasmid contained predominantly point mutations, whereas those derived from untreated plasmid contained predominantly gross alterations. A second shuttle vector carrying supF in a different position (pZ189) was obtained and the human 293 cell line was used. With this system the background frequency of supF mutants was lowered to 1.4×10^{-4}. The number of adducts required to reduce the bacterial transformation activity of the plasmid was the same for either carcinogen, but the frequency of supF mutants formed per adduct in 293 cells was 3.5-fold higher for BPDE than for 1-NOP. DNA sequence analysis of independent mutants derived from BPDE- or 1-NOP-treated plasmids showed that 60 out of 86 BPDE-induced mutants and 51 out of 60 1-NOP-induced mutants contained base substitutions. The majority involved G·C pairs, with 60% of these representing G·C $\rightarrow$ T·A transversions. Examination of the location of the point mutations among the 85 base pairs in the structure of the supF tRNA revealed that the progeny of the 1-NOP-treated plasmid had five hot spots, and those of the BPDE-treated plasmid had eight hot spots, only two of these hot spots were common for both agents.

To

my parents, Chin-Chieh and Shu-Huey Yang;
and
my husband, Jiunn-Fwu Lee

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ABBREVIATIONS

AAAF	2-(N-acetoxy-N-acetylamino)fluorene
amp	ampicillin gene
aprt	adenine phosphoribosyltransferase gene
BP	benzo[a]pyrene
BPDE	(±)-7β ,8α -dihydroxy-9α ,10α -epoxy-7,8,9,10-tetrahydro benzo[a]pyrene
BPV	bovine papillomavirus
BrdU	bromodeoxyuridine
dhfr	dihydrofolate reductase gene
DNP	dinitropyrene
EBNA-1	Epstein-Barr virus nuclear antigen 1
EBV	Epstein-Barr virus
EMS	ethyl methanesulfonate
ENU	N-ethyl-N-nitrosourea
galK	galactokinase gene
gpt	xanthine (guanine) phosphoribosyltransferase gene
hprt	hypoxanthine phosphoribosyltransferase gene
HSV	herpes simplex virus
IPTG	isopropyl β-D-thiogalactoside
lacI	a gene coding for the repressor of the lactose operon
L-N	Lesch-Nyhan
MNU	N-methyl-N-nitrourea

1-NOP	1-nitrosopyrene
1-NP	1-nitropyrene
PAH	polycyclic aromatic hydrocarbon
(6-4) py-C	(6-4) pyrimidine-cytosine
py-py	pyrimidine-pyrimidine
supF	a gene coding for a tyrosine suppressor tRNA
SV40	simian virus 40
tk	thymidine kinase gene
UV	ultraviolet
X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactoside
XP	xeroderma pigmentosum

INTRODUCTION

The recent discovery that some human cancers are associated with single point mutation in the ras oncogene family (Reddy et al., 1982; Tabin et al., 1982; Sukumar et al., 1983; Fujita et al., 1984) and that specific oncogenes can be activated through specific mutational changes by chemical carcinogens (Marshall et al.,1984; Vousden et al., 1986) enhanced interest in the molecular mechanisms by which mutations arise and in the role of mutation in initiating genetic disease and cancer. Attempts to deduce the nature of the molecular machanisms by which carcinogens induce mutations in mammalian cells have been hampered by the inability to isolate and analyze newly mutated genes at the sequence level. However, the development of shuttle vectors, i.e., plasmids carrying a defined target gene and capable of replicating in mammalian cells and also in bacteria, provide a solution to this problem (Calos et al., 1983; Razzaque et al., 1983; Sarkar et al., 1984; Lebkowski et al., 1985; Seidman et al., 1985; Bredberg et al., 1986; Drinkwater and Klinedinst, 1986; Hauser et al., 1986; Lebkowski et al., 1986). After fixation of mutations by replication of the vector in mammalian cells, mutants can be efficiently detected in bacteria and amplified for subsequent molecular analysis.

The shuttle vector approach was first demonstrated in simian cells, using vectors based on simian virus 40 (SV40). The use of these vectors has been limited by a high spontaneous mutation frequency (1%

to 10%) associated with the transfection process rather than arising during replication, and also by an inability of the vectors to replicate efficiently in mammalian cells (Razzaque et al., 1983; Ashman and Davidson, 1984; Lebkowski et al., 1984; Miller et al., 1984; Razzaque et al., 1984; Chakrabarti et al., 1985). Both of these problems were solved by the discovery by Calos and her associates (Lebkowski et al., 1984) that the human cell line 293 (Graham et al., 1977) replicates SV40-based shuttle vectors extremely well with a relatively low spontaneous mutation frequency. Another approach taken by Seidman et al. (1985) to minimize the high spontaneous mutation frequency was to decrease the possibility of recovering mutants containing gross rearrangements by the judicious design of a shuttle vector in which the target gene is strategically located between two genes essential for recovery of the vector in bacteria, i.e., the gene coding for ampicillin resistance and the bacterial origin of replication.

The well-studied carcinogen, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) is a major reactive metabolite of the widely distributed enviromental carcinogen benzo[a]pyrene (BP), an agent produced primarily by incomplete combustion processes (Weinstein et al., 1976). 1-Nitrosopyrene (1-NOP) is a partially reduced metabolite of 1-nitropyrene (1-NP), a carcinogen which accounts for 20% to 30% of the "direct-acting" mutagenicity of diesel emission particles (Rappaport et al., 1980; Rosenkranz, 1982; Schuetzle, 1983). Both BPDE and 1-NOP have been shown to form tumors in animals (Buening et al., 1978; Wislocki et al., 1986) and to induce mutations in bacteria (Mermelstein et al., 1981; Eisenstadt et al., 1982; Howard et

al., 1983; Heflich et al., 1985) and in mammalian cells (Yang et al., 1980; Patton et al., 1986). However, the mutational specificity induced in mammalian cells by these two compounds has not been determined. Both BPDE and 1-NOP form covalently bound adducts in DNA pricipally with guanine (Weinstein et al., 1976; Meehan et al., 1977; Heflich et al., 1985). 1-NOP binds to guanine at the C8 position (Heflich et al., 1985), whereas BPDE forms its pricipal guanine adduct at the N^2 position (Weinstein et al., 1976). Since these guanine adducts are located in very different position in the DNA helix and only the N^2 position is involved in the base pairing part of the molecule, the mechanism of molecular mutagenesis by these compounds may be different.

This thesis was undertaken (1) to investigate the specific kinds of mutations at the sequence level induced by two structurally-related carcinogens, i.e., BPDE and 1-NOP, when a shuttle vector replicates in mammalian cells; (2) to determine their location in the DNA sequence of the target gene and to see if these two compounds induce the same mutational spectrum; (3) to investigate if these two compounds have the same biological effectiveness, i.e., to compare the ability of their DNA adducts to interfere with bacterial transformation and to induce mutations during replication of plasmids in human cells.

Chapter I of the thesis reviews the background literature bearing on these questions. Chapter II describes fundamental work I carried out to set up the shuttle vector system for such mutagenesis studies. Chapter III consists of a manuscript published in the Notes section of the March 1987 issue of Molecular and Cellular Biology 7:1267-1270 which describes my work on mutations formed when p3AC containing

adducts of BPDE replicated in COS7 monkey cells. Chapter IV consists of a manuscript published in the June 1987 issue of Proceeding of the National Academy of Sciences U.S.A. **84**:3787-3791 which describes the research I carried out to determine the specific kinds of mutations induced when vector pZ189 containing BPDE adducts replicated in 293 cells, as well as their location in the supF gene. Chapter V describes comparable work carried out with vector pZ189 treated with 1-NOP and compares the results obtained with 1-NOP with those I found using BPDE. The format used for Chapter V is that of a manuscript to be submitted to Molecular and Cellular Biology as soon as I have completed experiments comparing the rate of excision repair of 1-NOP and BPDE-induced DNA adducts in the 293 cell line.

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CHAPTER I

LITERATURE REVIEW

A. Role of Polycyclic Aromatic Hydrocarbons and Their Nitrated Derivatives in Mutagenesis and Carcinogenesis

Polycyclic aromatic hydrocarbons (PAH) are environmental contaminants which are metabolized in a wide variety of species to reactive electrophiles that bind to cellular contituents and lead to mutations and cancer (Miller and Miller, 1974; Sims and Grover, 1974). Examples include pyrene, fluoranthene, benzo[a]anthracene, chrysene, benzo[e]pyrene, benzo[h,g,i]pyrene, coronene, and benzo[a]pyrene (BP). The most extensively studied PAH is BP which is ubiquitous in the environment because it results from the incomplete combustion of organic materials. It has been estimated that about 1300 tons of the carcinogen BP are released into the environment of the United State each year from sources such as heat and power generation, refuse buring, and coke production (National Academy of Science Reports, USA, 1972).

The nitrated PAH are also found in the environment, e.g., in diesel emission particles, fly ash, photocopier fluids, cigarette smoke, and emissions of fuel burners (Rosenkranz et al., 1980; McCoy and Rosenkranz, 1982; Rosenkranz, 1982; Tokiwa et al., 1985). Examples include 1,3-, 1,6-, and 1,8-dinitropyrene (DNP), 7-nitrobenzo[a]anthracene, 6-nitrochrysene, 4-nitropyrene, 6-nitrobenzo[a]pyrene, and 1-nitropyrene (1-NP). The predominant nitroarene in environmental samples appears to be 1-NP which is

produced in various combustion processes, including incomplete burning of diesel (Rosenkranz, 1982). 1-NP has been shown to account for 20% to 30% of the "direct-acting" mutagenicity of diesel emission particles (Rappaport et al., 1980; Rosenkranz, 1982).

The principal reactive metabolite of BP is the anti isomer of (±)BP-7,8,-diol-9,10-epoxide (BPDE) which binds to DNA mainly at the N^2 position of guanine (Figure 1a), with minor binding to adenine (Weinstein et al., 1976; Meehan et al., 1977). The reactive metabolite of 1-NP, formed by nitroreduction to the partially reduced intermediate metabolite, 1-nitrosopyrene (1-NOP), followed by further reduction to N-hydroxy-1-aminopyrene, binds covalently to DNA predominantly the C8 position of guanine (Heflich et al., 1985) (Figure 1b). The kinds of tumors formed by the parent carcinogens differ and since the DNA adducts they form with guanine are located in different positions in the DNA helix, the molecular mechanisms of mutagenesis by these two carcinogens and their biological effectiveness may differ significantly.

1. Metabolic activation of benzo[a]pyrene and 1-nitropyrene

Neither BP nor NP bind directly to DNA, RNA or proteins. They need to be activated to reactive metabolites through enzymatic metabolism. BP requires metabolic activation by the microsomal enzymes to exert several biological effects including cytotoxicity, mutagenicity, and carcinogenicity. BP was found to be metabolized to arene oxides, phenols, quinones, dihydrodiols, and diolepoxides (Yang et al., 1978). These metabolites were resolved by high-pressure liquid chromatography (Yang et al., 1978). The pathways of BP metabolism catalyzed by rat

Figure 1. Structure of the major BPDE-DNA adduct and 1-NOP-DNA adduct. a. BPDE-N^2-guanine adduct. From Weinstein *et al*. (1976), Science 193, 592-595. b. 1-NOP-C8-guanine adduct.

liver microsomes are summarized in Figure 2 (Yang et al., 1977). Studies of the metabolism-induced binding of BP and its metabolites to DNA have provided evidence that 7,8-diol-9,10-epoxides of this carcinogen are responsible for most of these bound adducts (Borgen et al., 1973; Sims et al., 1974). The ultimate metabolites of BP, the 7,8-diol-9,10-epoxides, exist as a pair of diastereomers in which the 7-hydroxyl group is either cis (syn form isomer) or trans (anti form isomer) to the 9,10 epoxide. Each diastereomeric 7,8-diol-9,10-epoxide can be resolved into a pair of optical enantiomers (Yagi et al., 1977). The structures of these four compounds are shown in Figure 3. The form studied in this thesis (Chpater III and IV) is the racemic mixture of the anti isomers, i.e., (±)-7β, 8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene which I abbreviate as BPDE.

In mammalian cells and bacteria, 1-NP is predominantly activated metabolically through nitroreduction. Reduction of the nitro moiety of NP appears to be a critical step in its metabolic activation since mutation assays conducted with nitroreductase-deficient strains of Salmonella typhimurium show much lower reversion frequencies than those obtained with nitroreductase-proficient tester strains (Wang et al., 1980; McCoy et al., 1981). The scheme proposed by Heflich et al. (1985) for the reductive metabolism of 1-NP to a derivative capable of binding to DNA is illustrated in Figure 4. The rate limiting step is enzymatic reduction to an intermediate product, 1-NOP (Heflich et al., 1985). The observation that 1-NOP forms the same DNA adduct as 1-NP and is more mutagenic than 1-NP in the Salmonella typhimurium strain TA1958 (Heflich et al., 1985; Beland et al., 1986) as well as in human diploid fibroblasts (Patton et al., 1987), suggests that 1-NOP is an

Figure 2. Mechanism of enzymatic activation of BP to BP 7,8-diol-9,10-epoxides. Abbreviations: MFO, mixied-function oxidases; EH, epoxide hydratase. From Yang et al. (1977), Science 196, 1199-1201.

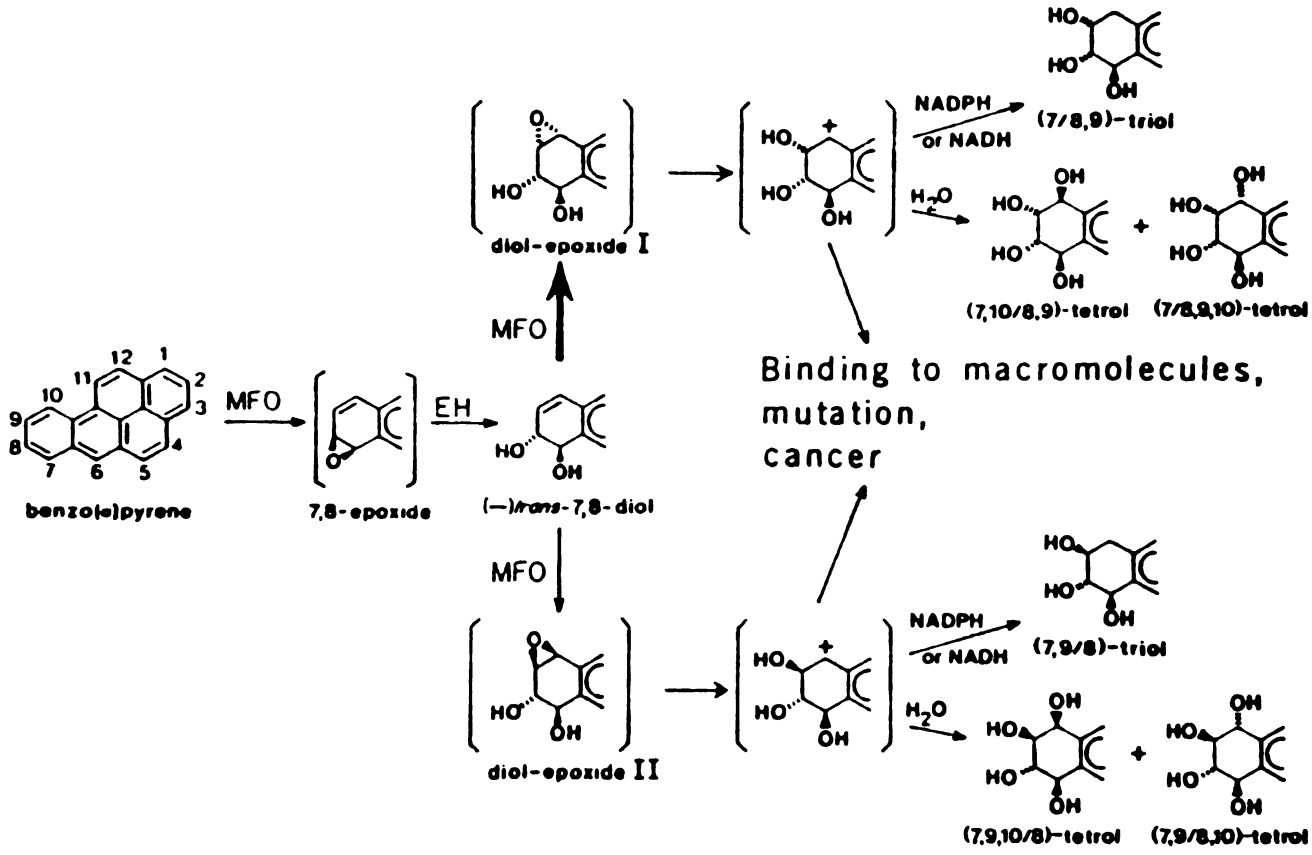
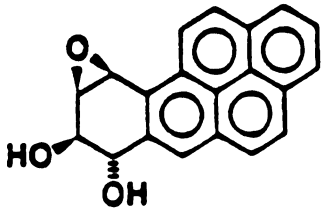
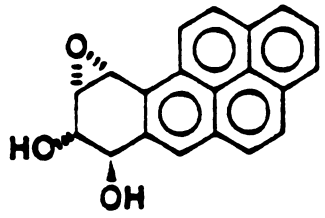

benzo[a]pyrene
MFO
7,8-epoxide
EH
(−)trans-7,8-diol
MFO
diol-epoxide I
MFO
diol-epoxide II
NADPH
or NADH
(7/8,9)-triol
H2O
(7,10/8,9)-tetrol
(7/8,9,10)-tetrol
Binding to macromolecules,
mutation,
cancer
NADPH
or NADH
(7,9/8)-triol
H2O
(7,9,10/8)-tetrol
(7,9/8,10)-tetrol

Figure 3. Structure and nomenclature of four isomers of benzo[a]pyrene diolepoxide. From Yang et al. (1978), in: Polycyclic Hydrocarbons and Cancer, Vol. I (Gelboin and Ts'O, eds.), Academic Press, pp. 205-231.





r-7, *t*-8-dihydroxy-*t*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene

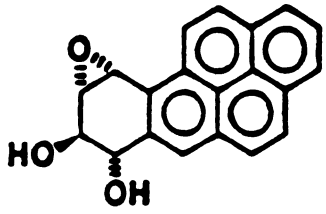
diol epoxide I

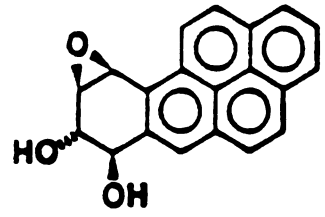
anti isomer

(±)7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene

(±)7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene

diol epoxide 2





r-7, *t*-8-dihydroxy-*c*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene

diol epoxide II

syn isomer

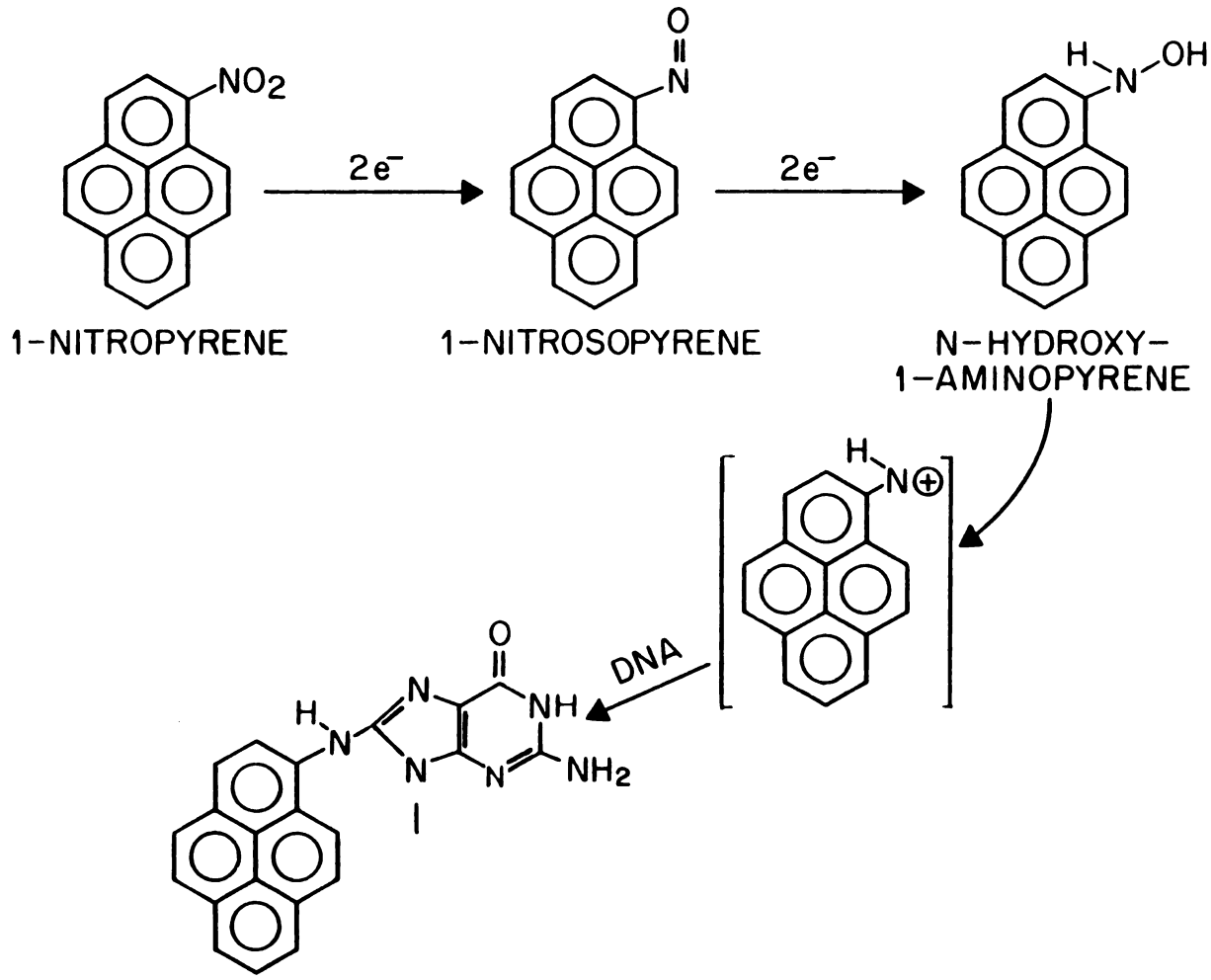
(±)7α,8β-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene

(±)7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene

diol epoxide 1

Figure 4. The reductive metabolism of 1-nitropyrene. From Patton et al. (1987), in: Polynuclear Aromatic Hydrocarbons: A decade of progress, (Cooke and Dennis, eds.), Battelle Press, pp. 678-698.

ACTIVATION OF 1-NITROPYRENE



intermediate in the mutagenic activation of 1-NP. Those studies indicate that the critical first step, enzymatic reduction to 1-NOP, is followed by a subsequent reduction to N-hydroxy-1-aminopyrene and the acid-catalyzed formation of a nitrenium intermediate which reacts readily with DNA. 1-NOP is the metabolite of 1-NP studied in this thesis (Chapter V).

2. DNA lesions produced by reactive metabolites of benzo[a]pyrene and 1-nitropyrene

It is now widely accepted that the covalent interactions of carcinogens with cellular macromolecules, particularly DNA, are essential initial steps in the process of mutagenesis and carcinogenesis (Miller and Mliier, 1974; Rajalakshmi et al., 1982; Weisburger and Williams, 1982). Studies by Sims et al. (1974), Ivanovic et al. (1976), Meehan et al. (1976), Weinstein et al. (1976), and Jeffrey et al. (1977) have indicated that the anti isomer of the 7,8-diol-9,10-epoxide of BP is the molecular species responsible for the major covalent binding of metabolized forms of BP to nucleic acids in hamster embryo cells, bovine, and human bronchial explants. Meehan et al. (1976) showed that treated with DNA in vitro, 92% of the total stable covalent adducts formed at deoxyguanosine, 5% at deoxyadenosine, 3% at deoxycytidine, and 0% at deoxythymidine. These investigators also studied the asymmetric binding to double-stranded DNA of the two stereoisomers of the 7,8-diol-9,10-epoxide of BP and showed that the (+)-enantiomers contribute most of the binding, e.g., 90% of deoxyguanosine adducts corrresponded to adduct formation with the (+) and and only 10% were with the (-) enantiomers. In contrast, only one

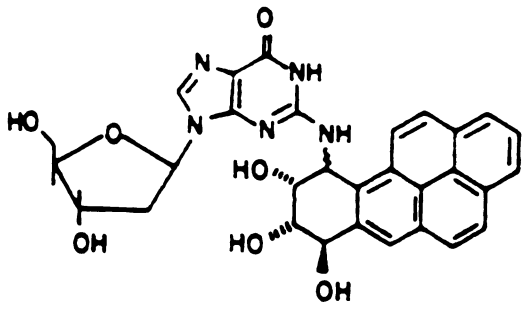
deoxyguanosinie adduct was formed using BP itself activated by a microsomal system. Straub et al. (1977) further isolated seven distinct products from the reaction of the racemic mixture of the anti isomer of the 7,8-diol-9,10-epoxide (BPDE) with thymus DNA and determined the structure of six of these products. The structure of these guanine, adenine, and cytosine adducts is shown in Figure 5a, b, and c. Each of these structures represents two diastereomers (each of the two enantiomers of BPDE reacted with the enantiomeric deoxyribonucleoside). Those adducts are formed between the C-10 position of the BPDE and the N^2 position of guanine, the N^4 position of adenine, and the N^4 position of cytosine. The major adduct corresponds to the binding of BPDE through the C-10 position to the N^2 position of guanine.

Minor adducts of BPDE at the O^6 and N7 position of guanine have been identified (Osborne et al., 1978; 1981). The minor adduct at the N7 position of guanine (Figure 5d) was spontaneously lost from the DNA within a few hours and resulted in the formation of alkali-labile apurinic sites (Osborne and Merrifield, 1985). It was found that the O^6 and N7 guanine products were derived mainly from reaction of the (-)-enantioner of the anti isomers of the 7,8-diol-9,10-epoxide of BP (Osborne et al., 1981). Unstable BPDE adducts which cause single strand breaks were detected in supercoiled DNA at neutral pH (Gamper et al., 1977), but this strand scission represents less than 1% of the DNA modification by BPDE.

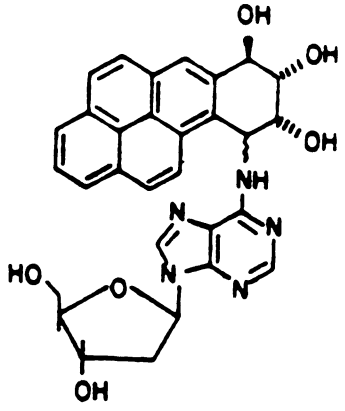
Howard et al. (1983) identified one major and two minor adducts of 1-NP when it was reacted with calf thymus DNA in the presence of a metabolic activation system. The major adduct was characterized as

Figure 5. Structure of guanine-, adenine-, and cytosine-BPDE adducts. a. N^2-guanine adduct. b. N^4-guanine adduct. c. N^4-cytosine adduct. d. N7-guanine adduct. Figure 5a, b, and c adapted from Straub et al. (1977), Proc. Natl. Acad. Sci. USA: 74, 5285-5289. Figure 5d adapted from Osborne and Merrifield (1985), Chem.-Biol. Interactions: 53, 183-195.

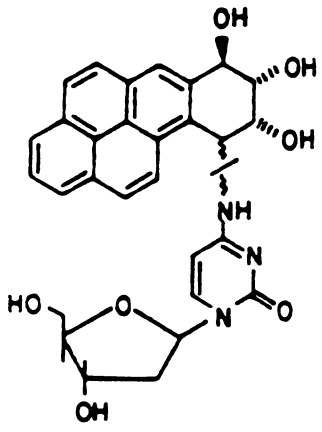
a.



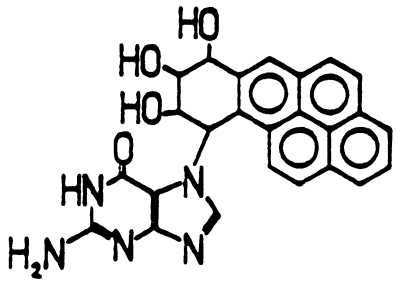
b.



c.



d.



N-(deoxyguanosin-8-yl)-1-aminopyrene (Figure 1). The minor adducts appear to be decomposition products of the major adduct. When calf thymus DNA was treated with 1-NOP in the presence of ascorbic acid as a source of reduction, the same major DNA adduct was formed as that formed by 1-NP in the presence of a metabolizing system (Heflich et al., 1985). In vivo studies of the formation of 1-NP and 1-NOP adducts showed that both compounds formed the same, single major DNA adduct, N-(deoxyguanosin-8-yl)-1-aminopyrene in bacteria (Howard et al., 1983; Heflilch et al., 1985) as well as in CHO cells (Heflich et al., 1986) and in human diploid fibroblasts (Beland et al., 1986; Patton et al., 1987).

3. Mutagenicity of reactive derivatives of benzo[a]pyrene, 1-nitropyrene, and dinitropyrenes.

The inherent mutagenic activity of BP derivatives has been exanmined in Salmonella typhimurium strains and in the Chinese hamster cell line V79. Of more than 30 BP metabolites and derivatives tested for mutagenic activity, the BPDE were the most potent mutagenic compounds in strains TA98 and TA100 and in V79 cells (Newbold and Brookes, 1976; Wislocki et al., 1976; Wood et al., 1976). Substantial differences in the mutagenic activities of the optically pure (+)- and (-)-enantiomers of the anti or syn isomers of the 7,8-diol-9,10-epoxide have been reported by Wood et al. (1977). In V79 cells, (+)anti-isomer was the most mutagenic for the four isomers. In contrast, (-)syn-isomer was a more potent mutagen than the other three optical isomers in Salmonella typhimurium.

Some nitropyrenes or dinitropyrenes are powerful mutagens, while

others are devoid of activity. The result depends on the type of bacteria or mammalian cells used for mutagenesis assay. Although 1-, 3-, and 6-nitropyrene and the three dinitropyrenes (DNP), 1,3-, 1,6-, and 1,8-DNP are direct-acting mutagens in the mutagenicity assay, 1,6-DNP and 1,8-DNP have more potential to induce revertants than does 1-NP. The mutagenicity of 1-NP is greatly reduced when tested in bacterial strains deficient in nitroreductase, suggesting that this enzyme is required for expression of 1-NP-induced mutagenicity. In contrast, 1,6-DNP and 1,8-DNP did not depend on the classical introreductase for maximal activity (Mermelstein et al., 1981; Rosenkranz and Mermelstein, 1983). In studies of the mutagenicity of nitropyrenes in cultured mammalian cells, 1-NP was active in inducing thioguanine resistant mutants in human HepG2 hepatoma cells, whereas 1,6-DNP and 1,8-DNP were inactive (Eddy et al., 1986). Conversely, in Chinese hamster V79 cells, (Takayama et al., 1983), CHO cells (Li and Dutcher, 1983), or lung fibroblasts (Nakayatsu et al., 1982), 1,6-DNP and 1,8-DNP exhibited maximal activity while 1-NP showed almost no activity. These findings suggest that the diverse enzymes involved in nitroreductions in various systems differ in their specificity and catalytic mechanism. Eddy et al. (1986) suggested that the biological activity of nitropyrenes and dinitropyrenes was correlated with the number of electrons involved in the rate-determining step of nitroreduction, e.g., for 1-NP, one electron transfer was involved in the reduction of the first nitro function; for 1,6-DNP and 1,8-DNP, two electrons were tranferred (Klopman et al., 1984).

4. Carcinogenicity of benzo[a]pyrene, benzo[a]pyrene diolepoxides, 1-nitropyrene, and dinitropyrenes

Benzo[a]pyrene forms tumors predominantly in the lung (Levin et al., 1978; Wislocki et al., 1986). The induction of pulmonary tumors in the newborn mouse demonstrated that BPDE is ultimate carcinogenic metabolite of BP (Kapitulnik et al., 1977; 1978). Buening et al. (1978) further tested the tumorigenic activity of each of the four optically pure isomers of the 7,8-diol-9,10-epoxides of BP on newborn mice. They found that the (+)anti-isomer had exceptional tumorigenicity, whereas BP and the other three optically pure isomers had no or little activity at a total dose of 7 or 14 nmol used. Bresnick et al. (1977) reported that BPDE is the most potent inducers of mouse skin epidermal hyperplasia of some 20 BP derivatives tested. In another different tumor model, the initiation-promotion system of tumorigenesis on mouse skin, used to evaluate the carcinogenic potential of BP and its derivatives, BPDE was less active than BP as tumor initiators (Slaga et al., 1976; 1977). This result may reflect the fact that BPDE is an extremely unstable compound. Slaga et al. (1979) further analyzed the skin tumor-intiating activity of the four isomers. They found that the (+)anti-isomer was the most active tumor initiator (60% as active as BP) among the four isomers. When the (+)anti-isomer was given in fractionated doses, the skin tumor-intiating activity increased and was comparable to that of BP. These results, together with the data of the mutagenesis studies with V79 cells (Huberman et al., 1976; Wood et al., 1977), indicate that the (+)anti-isomer is the ultimate carcinogenic metabolite of BP in mammalian cells.

Ohgaki et al. (1982) showed that 1-NP produced subcutaneous tumors at site of injection in 8 out of 17 male F344/DuCrj rats. However, Ohgaki reported later (quoted in a meeting report by Serres and Matsushima, 1986) that the batch of 1-NP used for the previous study had been contaminated with 0.2% 1,3-DNP, 0.3% 1,6-DNP, and 0.3% 1,8-DNP, the three dinitropyrenes were found to produce subcutaneous tumors at site of injection in ten out of ten rats. No tumors were found when the 1-NP experiment was repeated using a highly purified sample. The poor carcinogenicity of 1-NP in BALB/C mice was also reported by Tokiwa at that same meeting. The weak mutagenicity and carcinogenicity of 1-NP in rodents may related to the low ability of rodent cells to carry out the reduction of 1-NP to a reactive metabolite. In contrast, Hirose et al. (1984) showed the ability of 1-NP to induce malignant fibrous histiocytomas in ten out of 31 male and nine out of 32 female animals at sites of injection, and mammary gland tumors in 15 out of 32 femal newborn Sprague-Dawley derived CD rats. The ability of NP to induce lung tumors in A/J mice has also been shown by El-Bayoumy et al. (1984). Beland and his coworkers have reported that i.p. injection of 1-NOP at total doses of 2800 nmols per mouse induced 21% - 28% liver tumors in new born mouse, Liver tumors did not occur in females, and lung tumors were not induced in both male and female animals (Wislocki et al., 1986). These investigators also reported that 1-NOP caused liver tumors in 45% of the male and 9% of the females at 700 nmols per mouse by i.p. injection (Wislocki et al., 1986).

5. The specific kinds of mutations induced by BPDE and 1-NOP

Several investigators have examined the kinds of mutations induced by BPDE in bacteria, but few have studied this question in mammalian cells. In the Salmonella reverse mutation assay, BPDE caused a much higher mutagenic response in a missense mutation tester strain (TA100) than in a frameshift mutation tester strain (TA98) (Malaveille et al., 1977; Wood et al., 1977; Fahl et al., 1981; Aust et al., 1984). The most extensive studies of the specific kinds of base changes induced by BPDE in E. coli was carried out by Eisenstadt et al. (1982) who showed that BPDE significantly induces G·C → T·A transversions in the lacI gene of E. coli by genetic mapping. Using bacterial plasmid transformation and recombinant DNA techniques, Mizusawa et al. (1981a) treated a bacterial plasmid carrying a marker gene with BPDE and transferred this treated plasmid into various E. coli recipients to study the effect of BPDE on plasmid survival in bacteria and on the frequency of mutants. Agarose gel electrophoresis analysis of restriction endonucleotide HpaII digested mutant plasmids showed that 94% did not involve detectable alterations in the size of the DNA fragments, indicating that BPDE is a point mutagen. Limited data on the specific kinds of mutations induced at DNA sequence level by BPDE in bacteria plasmid have been reported. Mizusawa et al. (1981b) found that two BPDE-induced mutants exhibited T·A insertions and one contained a G·C base pair deletion. Chakrabarti et al., (1984) reported that three out of five BPDE-induced mutant plasmids had G·C base pair deletions, one contained a G·C → T·A transversion, and one had a A·T → G·C transition.

The specific kinds of mutations induced at the DNA sequence level

in mammalian cells have never been studied. King and Brookes (1984) showed that BPDE induced mutations in V79 Chinese hamster cells were predominantly point mutations as detected by DNA hybridization. The data from Aust et al. (1984) also indicate that BPDE is a point mutation mutagen because it does not cause gross alterations expected to completely inactivate the gene coding for elongation factor 2, which is involved in diphtheria toxin resistance.

Both 1-NP and 1-NOP induce revertants in Salmonella typhimurium frameshift tester strains TA1537, TA1538, and TA98 (Mermelstein et al., 1981; Heflich et al., 1985). No revertant has been found in strain TA1535 (a base substitution indicator) and only some revertants have been found in strain TA100 (a missense mutation indicator) when treated with 1-NP. Little is known about the kinds of mutations induced by 1-NP or 1-NOP in either E. coli or mammalian cells.

B. Systems Presently Available for Investigating Mutagenesis in Mammalian Cells at the Molecular Level

The molecular mechanisms responsible for mutational changes in mammalian calls are not well understood. In this decade, the development of recombinant DNA techniques has enabled researchers to identify mutational changes in genes by direct nucleic acid analysis. These molecular mutagenesis systems resolve the difficulties of previous indirect mutational analysis, i.e., detection of phenotypic changes or gene products. Some of these systems for studies of the molecular nature of spontaneous and induced mutations in mammalian cells have been reviewed by Lehmann (1985) and Thacker (1985). During the past two years, still more elegant systems for such molecular

studies of mutations have been developed (see below), making this an exciting era in which to investigate the specific kinds of mutations at the sequence level caused by specific mutagens and carcinogens.

The recent applications of these recombinant DNA techniques to analysis of spontaneous and induced mutations in cultured mammalian cells include: 1) mutations in endogenous mammalian genomic genes, 2) mutations in bacterial genes integrated into the mammalian genome, 3) mutations in viral vectors, 4) mutations in transiently replicating shuttle vectors, and 5) mutations in stably replicating shuttle vectors.

1. **Endogenous mammalian gene systems**

In these systems, the particular genes studied are those for which cloned cDNA sequences have been isolated. The entire genomic DNA is digested with restiction enzymes and the DNA fragments are electrophoresed. Southern blotting of these restiction fragments, followed by hybridization with the cloned cDNA or other probes, permits analysis of mutations in the structural genes. Deletions or insertions of genetic material result in alteration in the size of hybridizing restriction fragments. Base alteration mutations localized to restriction endonucleoase recognition sites in the particular gene can also be detected, since the nucleotide alterations caused by the mutations lead to loss or gain of the recognition sites. Mutant endogenous genes can be further analyzed by sequencing the base changes causing the alterations of the recognition sites (Nalbantoglu et al., 1987).

The amount and the size of the mRNA in the mutant cells can also

be analysed by transfer of the RNA from mutant cells onto filter papers, followed by hybridization with the cloned cDNA probe (Northern blotting). Changes in the blotting pattern can reveal alterations in gene expression.

a. Studies with the hypoxanthine phosphoribosyltransferase (*hprt*) gene

The successful production of cDNA clones of the hypoxanthine phosphorisyltransferase (*hprt*) gene (Brennand *et al.*, 1982; Konecki *et al.*, 1982; Jolly *et al.*, 1983) has enabled molecular studies of mutations in this locus. The mouse gene is ~33kb and contains nine exons varying in length from 18 bases to 593 bases (Melton *et al.*, 1984). These is close homology of the HPRT protein sequence of mouse, hamster and human (Konecki *et al.*, 1982) and the organization of mouse and human *hprt* gene (Yang *et al.*, 1984), suggesting strong evolutionary conservation of this gene. In the process of characterizing the *hprt* gene of a mouse cell line, Melton *et al.* (1984) reported that the spontaneous *hprt* mutant used for seletion of an overproducing revertant contains a single base transition of G → A in exon 8. Fuscoe *et al.* (1983) analysed ten spontaneous and nine UV-induced *hprt*$^-$ mutants of V79 Chinese hamster cells. Only one spontaneous and one UV-induced mutant exhibited large deletions in the *hprt* gene when hybridized to 1.1 kb mouse cDNA or 1.3 kb hamster cDNA probe. Fenwick *et al.* (1984) also studied a *hprt* mutant of Chinese hamster cells and its *hprt*$^+$ revertants with a cloned cDNA for *hprt*. This mutant line may contain a point mutation because no alterations in restriction patterns were observed. Some of the spontaneous revertants produced a much stronger hybridizing signal, indicating that the gene had been

amplified up to ten to twenty times. This resulted in a corresponding overproduction of mRNA. Overproduction of the defective gene product was presumably responsible for phenotypic reversion.

In a related study, King and Brookes (1984) found that each of eleven 8-azaguanine resistant, cross-reacting material-negative Chinese hamster cell mutants induced by BPDE showed no detectable alteration in gene structure when six different restriction enzyme digested mutant DNA fragments were probed with the 1.3 kb hamster cDNA. However, four of these mutants showed much less amount of hprt mRNA than did wild type cells. Two of these four mutants also showed a size reduction by about 300 bases. The truncated mRNA was suggested to arise from incorrect processing, perhaps by point mutation of a splice site. Their results suggest that BPDE functions primarily as a point mutagen.

In humans, complete deficiency of HPRT enzyme leads to the Lesch-Nyhan (L-N) syndrome and partial deficiency is associated with gouty arthritis. This fact has also drawn attention to this gene. Using mouse cDNA probes, Yang et al. (1984) examined DNA from cells of 28 untreated L-N patients. These DNA samples were digested with four different restriction enzymes and analysed by Southern blotting using a full-lenth mouse hprt cDNA probe. These investigators found that 23 L-N patients out of the 28 surveyed did not show major hprt gene alterations. Assuming that the entire human gene is organized similarly to the mouse gene, and using cDNA probes encoding only parts of the gene, it was possible to show that each of the five L-N patient with an altered hprt gene carried a different mutation. Three had deletions at different positions, one had alterations in exons 4, 5 and/or 6, and one had a suggested duplication involving in exons 2 and

3.

b. **Studies with the Adenine Phosphoribosyltransferase (aprt) gene**

Adenine phosphoribosyltransferase (aprt) gene is a particularly attractive locus for analysing endogenous mutational events. Since this gene is located on autosomal chromosome, the interpretation of molecular data is complicated by the presence of two copies of the gene. However, a strain hemizygous for aprt has been identified (Bradley and Letovanec, 1982; Nalbantoglu et al., 1983), making it possible to collect single-step spontaneous and induced mutants and allow mutagenesis studies in this locus. Furthermore, the relatively small size of aprt gene facilitated its cloning from the genomic DNA of CHO cells (Lowy et al., 1980): a 3.8 kb BamHI fragment contains all the sequences necessary to transform $APRT^-$ CHO cells to $APRT^+$. This allows, in many instances, localization of mutational events in vivo to restriction endonuclease sites and rough mapping of deletion or insertion termini (Nalbantoglu et al., 1983). Another advantage of using aprt as a model for the study of gene variation in mammalian cells is that it is a true endogenous gene with the low mutational rate inherent to such loci (4×10^{-8} per cell per generation; Nalbantoglu et al., 1987). However, an odd feature of these loci is that the rodent aprt genes are not good probes for the human aprt gene in Southern blot hybridizations (Stambrook et al., 1984). Furthermore, the DNA sequence analyses of these loci were limited to mutants which lost restriction endonuclease sites. This limitation eliminates the possibility that mutations might exist outside these regions, and thus the true mutational spectrum at the DNA sequence of this locus cannot be

obtained.

Meuth and his colleagues (Meuth and Arrand, 1982; Nalbantoglu et al., 1983: Goncalves et al., 1984) analyzed mutations induced in the hamster aprt gene by ethyl methanesulfonate (EMS). These studies showed that most mutants induced by EMS in CHO cells have unaltered restriction fragment patterns, suggesting that EMS is a point mutagen. Most of the spontaneous mutations were also point mutations, but a small number of deletions and an insertion were also detected. A number of these mutations were localized to restriction endonuclease recognition sites in the aprt gene, since the nucleotide alterations produced by the mutations led to the loss or gain of the site. Nalbantoglu et al. (1987) further cloned those spontaneous aprt genes and sequenced the base changes causing the alterations of the sites. Of the 12 mutant genes analyzed, five contained single base pairs transitions (both G·C → A·T and A·T → G·C), two exhibited transversions (G·C → T·A and A·T → T.A), one showed multiple mutations, i.e., a G·C → T·A transition next to a single base pair insertion, and four contained more complex changes, involving small deletions or duplications.

c. Studies with the dihydrofolate reductase (dhfr) gene

As for hprt, the dhfr genes have proved to be considerably larger than their mRNA: in the mouse ~31 kb (Crouse et al., 1982), Chinese hamster ~25 kb (Carothers et al., 1983), and human ~30 kb (Chen et al., 1984). The genes from these species are similar in being split into six exons with conservation of intron-exon junctions, but having some divergence in intron length and sequence. Several different-sized mRNA

coding for DHFR have been found in mouse, hamster, and human cells (Crouse et al., 1982; Carothers et al., 1983; Morandi et al., 1982).

Graf and Chasin (1982) analyzed γ-irradiation induced mutations at the dhfr locus in a presumed heterzygote CHO cells using a mouse cDNA probe. Two out of nine mutants analyzed contained altered restriction patterns consistent with a deletion, insertion or rearrangement.

In these endogenous mammalian gene systems, it is obviously useful to select cell lines lacking one copy of autosomal genes to rapidly and unambiguously identify mutations in the remaining copy (e.g., aprt and dhfr seletion). But the use of these systems to investigate point mutations is difficult. The use of a sufficiently large number of restriction enzymes to detect if a base substitution or frameshift is involved is very time-consuming and expensive, espacially for a large gene. Cloning of mutants into bacteriophage makes it possible to investigate mutation at the level of base alterations by sequencing. However, this method is not widely used because of the time and labor involved.

2. Integrated exogenous gene systems

The above mentioned difficulties of analyzing native genes have been circumvented by introducing small cloned genes as targets for mutagenesis. These genes can be integrated into the mammalian cell genome or introduced as autonomously replicating genes. The use of genes, coding for selectable markers,and integrated into genomic DNA for mutation studies has the advantage that the genes behave like chromosomal DNA with respect to replication and mutagenesis. The

target genes, e.g., xanthine (guanine) phosphoribosyltransferase (gpt), supF, and thymidine kinase (tk), are carried in vectors, such as a SV40-based plasmid (Ashman and Davidson, 1984; Tindall et al., 1984) or phage vector (Glazer et al., 1986) or retroviral vector (Ashman and Davidson, 1987). In most of these systems (gpt and tk), the target genes are transfected into a deficient cell line (TK^- or $HPRT^-$) and integrated into genomic DNA. Transformants containing the integrated gene are treated with mutagens and selected in a special medium for the mutation assay. For example, Perucho et al. (1980) and Robins et al. (1981) have used the Herpes simplex virus (HSV)-tk gene to transform TK-deficient mouse, human, or rat cells, and studied mutations of HSV-tk transformants by selection in medium containing bromodeoxyuridine (BrdU). They reported that most of the spontaneous mutation consisted of various-sized deletions of the introduced HSV-tk gene. This phenomenom may be the result of the instability of newly-introduced genes (Scangos et al., 1981).

a. Studies with the gpt gene

Thacker et al. (1983) reported that most of mutants they detected after introducing vector DNA into genome were due to complete loss of the integrated target gene. They then established a hamster V79 cell line containing a single gpt gene and expressing gpt stably even when maintained for generations in non-selective conditions. These cells showed a spontaneous gpt^- mutant frequency of 5×10^{-4}. The frequency of mutations induced in the gpt gene by x-rays and EMS was about 20-fold high than that found with the endogenous hprt gene in V79 cells. However, the target gene of the majority of these gpt^- mutants also

proved to be completely deleted.

Ashman and Davidson (1987) have introduced a retroviral shuttle vector containing the gpt gene into a hprt$^-$ mouse cell line by infection. They isolated a stable cell line which contains a single copy of the vector integrated into chromosomal DNA in a proviral form. Cells with spontaneous mutations in the gpt gene were selected by their resistance to 6-thioguanine and then were fused with COS cells for recovery of the mutant genes. The majority (29/43) of the mutants contained deletions with 19 out of 29 containing a deletion of three base pairs which resulted in the "in frame" loss of an aspartic acid codon. Eleven mutants contained single base substitutions, viz., six transversions and five transitions. These investigators found no obvious preference for any particular type of base substitution among the spontaneous point mutants sequenced. They also studied mutations induced by EMS and BrdU. They reported that both chemicals induced mutants and that based on the restriction enzyme analysis, the mutants contained 90% putative point mutations.

b. Studies with the supF gene

Glazer et al., (1986) developed a lambda phage vector carrying the neomycin resistance gene which allows selection for stable integration of the vector into the mammalian genome. The vector also carries a small gene, supF, as a target for mutations. They transfected this vector into a mouse L-cell line and established stable lines with multiple copies of this lambda phage vector integrated into mouse genomic DNA. They efficiently rescued viable phage from high molecular weight mouse cellular DNA using lambda in vitro packaging

extracts and found a negligible background of mutant phage (0/54,605). With this system, they exposed mouse cells carrying multiple copies of the lambda vector in the genome to 254 nm ultraviolet (UV) light. Of 78,510 phage were rescued, eight supF mutants were found. DNA sequence analysis of the mutants suggests that the primary site of UV mutagenesis in mammalian cells was at pyrimidine-cytosine sequences, and that the most frequent mutation was a G·C → A·T transition.

These recently developed integrated vector systems (Glazer et al., 1986; Ashman and Davidson, 1987) allow one to recover the integrated mutated genes and the integrated gene behaves somewhat like chromosomal DNA with respect to replication. The major advantage of these systems over the endogenous gene systems is that the small defined target genes (supF; gpt) enable rapid sequencing. Thus the molecular mechanism induced by mutagens at the base changes level can be easily studied.

3. Viral vector systems

Animal virus, such as HSV and SV40, can themselves be used as the target for mutations in mammalian cells. Those viruses are useful in such a study because they replicate as minichromosomes in mammalian host cells and the mutants can be rescued from the cells, mapped and sequenced. Dasgupta and Summers (1978) have made use of the genetic system involving the TK locus of HSV-1 to reveal that an "error-prone" inducible UV-reactivation phenomenon exists in mammalian cells. Although they did not determine the molecular nature of the mutants at that times, it is posible to analyse those tk^- mutants at the molecular level at present.

Bourre and Sarasin (1983) used SV40 virus as a biological probe to

study the mutagenic effect of UV-irradiation at the molecular level. They studied reversion of the tsA58 locus (a thermosensitive SV40 mutant of the large T antigen) in SV40 virus which had been UV-irradiated *in vitro*. Monkey kidney cells were infected with UV-irradiated tsA58 virus. Revertants of tsA58 were selected by growth of the virus at the nonpermissive temperature. Then the reversion sites were analysed by using the marker rescue technique and DNA sequencing. Of 16 mutations sequenced, all were localized opposite a possible UV-induced pyrimidine-pyrimidine lesion, suggesting targeted mutagenesis of UV. The advantage of this system is that the spontaneous reversion rate of the tsA58 is low ($\sim 10^{-6}$). One major disadvantage of using this system is that frameshift mutations cannot be detected because under their conditions, any frameshift mutation leads to the production of an inactive T antigen which is lethal for the virus.

Using the same system, Gentil *et al*., (1986) studied mutations induced by 2-(N-acetoxy-N-acetylamino)fluorene (AAAF). In more than 60% of independently isolated mutants, the molecular analysis of AAAF-induced revertants revealed a hot spot for a base substitution mutation not locaized opposite a major DNA lesion. These investigators hypothesized that a specific DNA structure, stablized by an AAAF adduct, is responsble for the hot spot. In a bacterial forward mutation system, 90% of AAAF-induced mutations were frameshifts (Koffel-Schwatz *et al*., 1984). The inability of the viral assay to be used to detect frameshift mutations eliminates the chance of making comparisons with the AAAF-induced mutations data in the prokaryotic system.

4. Transiently Replicating Shuttle Vector Systems

Mutagenesis studies involving endogenous genes are not ideal for analyzing mutations at the base-pair changes level. Shuttle vectors, however, provide rapid and powerful tools for the study of mutagenesis at the DNA sequence level in cultures mammalian cells. A shuttle vector is a plasmid DNA that is capable of replicating in mammalian cells and in bacteria. In many instances, such shuttle vectors contain the origin of replication and large T-antigen from SV40 which allows transient replication in mammalian cells, the bacteria origin of replication for propagation in bacteria, a drug resistant gene (e.g., the gene for ampicillin resistance), and a variety of bacteria genes as targets for mutagenesis, e.g., supF, galK (a gene coding for a galactokinase), and lacI (a gene coding for the repressor of the lactose operon). The vectors are transfected into permissive mammalian cells and allowed to replicate in the nucleus for several days. Mutagenesis takes place during this time. The progeny vectors are then extracted and separated from cellular DNA, and used to transform an appropriate indicator bacteria strain for mutant identification at the target gene. The mutation in the defined target genes can be easily analyzed by restriction mapping, genetic techniques (only for lacI gene), and DNA sequencing.

One particular advantage of the transiently replicating systems over the stably replicating systems (see part B.5) is that vectors can be treated with mutagens in vitro and allowed to replicate in a variety of cells which themselves have been treated or not treated with mutagens. Therefore, transiently replicating vectors are particularly useful for indirect mutagenesis studies, such as studies of SOS

inducible error-prone mutagenesis in mammalian cells.

a. High background mutant frequency of transfected vector DNA

A major problem recognized with the early developed shuttle vector systems was the very high "spontaneous" mutant frequency in the transfected vectors (0.2% - 10%) (Calos et al., 1983; Razzaque et al., 1983; Ashman and Davidson, 1984; Lebkowski et al., 1984; Sarkar et al., 1984). This mutant frequency was several orders of magnitude higher than the spontaneous rate for the same genes in E. coli of 10^{-5} - 10^{-6} (Miller et al., 1977). It was also much higher than the spontaneous mutation rate for genes in mammalian cells of 10^{-6} - 10^{-8} (Lewin et al., 1980).

Studies by Lebkowski et al. (1984) indicated that the high mutation frequency was due to damage incurred in vector DNA early after its entry into the mammalian cells, rather than during replication, since even nonreplicating molecules were subject to the effect. Other evidence in support of this hypothesis comes from the finding by Razzaque et al. (1984) that the mutation frequency did not increase during the replication period. It has been shown that transfection of DNA by $CaPO_4$ coprecipitation, DEAE-dextran, protoplast fusion, or electroporation, all led to elevated mutation frequencies (Calos et al., 1983; Razzaque et al., 1983; Lebkowski et al., 1984). The target gene itself was not causing the high mutation frequency (Calos et al., 1983). Both point mutations and gross rearrangements were detected (Calos et al., 1983; Lebkowski et al., 1984). It has been suggested that intracellular DNA damage is the cause of the formation of mutations in transfected DNA. If newly transfected DNA were subject to

attack by endonucleases, exonucleases, and ligases which are present in the mammalian nucleus, deletions could readily be generated (Miller et al., 1984; Razzaque et al., 1984)

Genetic mapping and DNA sequencing of the spontaneous point mutants obtained in the lacI gene during replication in COS7 cells showed that the majority of the point mutations were base substitutions (Miller et al., 1984). Of 93 independent base substitutions obtained in lacI gene during passage through COS7 cells, all occurred at G·C base pairs and were approximately equally divided between G·C → A·T transitions and G·C → T·A transversions. These investigators suggested that this specificity resulted from deamination of cytosine and depurination of guanine.

b. **Systems to minimize the high background frequency of mutants**

Several groups have made efforts to design other transiently replicating vector systems to achieve a lower background mutation frequency than that observed previously. Lebkowski et al., (1984) introduced a series of vectors into a variety of mammalian cells to characterize further the mutational fate of transfected DNA. They found that high mutation frequency appears to be the characteristic outcome of transfection of DNA into mammalian cells. However, they observed an approxiomately 10-fold lower mutation frequency when a SV40-based vector, pJYMib, or a BK-based vector (BK is a human papovavirus closely related to SV40), pBKib, were passaged in a human embroynic kidney cell line, designated 293. They also noted that replication of transfected vectors was more efficient in 293 cells. Their finding that efficient replication of shuttle vectors in 293

cells was accompanied by a relatively low background frequency allowed a shuttle vector system to be used successfully in this thesis for the study of mutations induced by BPDE and 1-NOP (see Chapter IV and V). Using 293 cells, Calos and her coworkers have reported on lacI mutants induced by UV (Lebkowski et al., 1985) and EMS (Lebkowski et al., 1986). Lebkowski et al. (1985) reported that UV predominantly induces point mutation mutants. The frequency of point mutations was increased 5-fold with 50 J/m^2 dose of UV. Using genetic mapping techniques to analyze nonsense mutations and DNA sequencing techniques to locate the missense mutations, they observed that UV specifically induced G·C → A·T transitions (81%). More than 90% of the UV-induced transitions involved pyrimidine-pyrimidine (py-py) sequences. Since the two major photoproducts caused by UV, the cyclobutane pyrimidine dimer and the (6-4) pyrimidine-cytosine (py-C) photoproduct, occur at py-py sequences, the location of the majority of the mutation opposite to these sequence argues that the UV mutagenesis in the human cells was targeted to premutational lesions. The base substitution specificity observed closely resembles that observed with UV light in E. coli (Coulondre and Miller, 1977; Miller, 1985), suggesting that human and bacterial cells respond similarly to damage from UV light. However, frameshifts were seen in E. coli (60%), but occurred very rarely in human cells (4%).

The base-pair changes induced in 293 cells by EMS were also determined using the identical lacI shuttle system. EMS induced predominantly point mutations which occurred at a frequency six times above background. Ninety-eight percent (53/54) of the lacI mutations derived from EMS-treated human cells were G·C → A·T transitions.

These results are similar to the data obtained for *lacI* in *E. coli* in which 98% (680/691) of the mutations were also G·C → A·T transitions (Coulondre and Miller, 1977). The major mutagenic lesion caused by alkylation agents is the O^6-alkyl guanine (Singer *et al.*, 1986). If the O^6-alkyl group is not removed by repair enzymes before replication, mispairing of the O^6-alkyl guanine with thymine can form, leading to a full G·C → A·T transition during the next round of replication. These results also demonstrated targeted mutagenesis operating in mammalian cells.

Seidman *et al.*, (1985) constructed a plasmid so as to minimize the recovery of these background deletions and to facilitate the detailed characterization of the induced mutations. Their vector, pZ189, contains the origin of replication and large T-antigen gene from SV40, as well as the small target gene, *supf*, strategically located between two genes essential for recovery of the plasmid in *E. coli*, i.e., the gene for ampicillin resistance and the bacterial origin of replication. Mutations containing base substitutions and small deletions or insertions in the region of *supF* gene can be recovered. However, large deletions and rearrangements in the region of *supF* gene that extend into either of the two flanking regions which are made up of genes essential for recovery of the plasmid in bacteria are not recovered. When this vector was allowed to replicate in a variety of mammalian cells, it exhibited a low relatively low frequency of spontaneous deletions (Seidman *et al.*, 1985; Bredberg *et al.*, 1986; Hauser *et al.*, 1986). The small size of the tRNA target gene permits rapid sequencing of the entire gene in a single opteration, and thus all kinds of mutations that eliminate the function of the suppressor tRNA

can be characterized. It has been shown that the supF gene is highly responsive to base changes. Among the 85 nucleotides making up the tRNA structure, base changes at any one of at least 63 positions results in a detectable phenotypic change (Celis and Piper, 1982; Bredberg et al., 1986; Glazer et al., 1986; Hauser et al., 1986; Yang et al., 1987a; 1987b).

Seidman et al. (1985) compared the replication efficiency of pZ189 in the CV1 monkey cell line to that of SV40 viral DNA. The results indicated that as much plasmid pZ189 DNA accumulated in the cells as SV40 viral DNA. High efficiency of replication of pZ189 has also been observed in SV40 transformed human fibroblast cells, including XP cells (Berdberg et al. 1986). This high efficiency of replication of pZ189 facilitates the recovery of plasmid DNA following transfection with plasmids partially inactivated by mutagen treatment. In addition, use of this vector allows a more detailed characterization of molecular defect that may underlie individual sensitivities to DNA damaging agents leading to increased risk of cancer and other consequences of mutagenesis. For example, elegant studies to reveal the UV-induced mutagenesis in mammalian cells have been done using this shuttle vector. Hauser et al., (1986) treated the vector with UV light at a dose of 500 J/m^2, then transfected this UV-irradiated vector into CV1 monkey cells. They assayed the mutations that formed during the vector replication in CV1 cells for 2 days. They found a 20-fold increase of mutant frequency above background. Most of the UV-induced mutations were point mutations (95%), primarily base substitutions. UV-induced base substitutions all involved G·C base pairs, despite the preference for photoproduct formation at TT sites. These investigators suggested

that the preference for G·C → A·T transition among UV-induced mutation reflects the fact that DNA polymerases reponsible for these mutations prefer to insert adenine opposite photoproducts in the DNA. They also reported that hot spots for UV mutagenesis did not correspond to hot spots for UV-induced photoproduct formation when determined by a DNA synthesis arrest assay. This conclusion was later supported by related studies by Brash et al. (1987).

In an extended study, the UV-irradiated vector was treated with E. coli photolyase prior to transfection so that pyrimidine cyclobutane dimers were removed selectively (Protić-Sabljić et al., 1986). Removal of 90% of pyrimidine cyclobutane dimers decreased the mutagenic activity frequency by 80%, suggesting that UV-induced cyclobutane pyrimidine dimers are mutagenic in mammalian cells. There were significantly fewer tandem double-base changes and G·C → A·T transitions after photoreactivation of tha DNA. These two alterations may indicate that cyclobutane dimers are principally responsible for these types of mutational changes and that (6-4) py-C photoproducts may be more likely to cause single base transversions.

Using this vector, Bredberg et al., (1986) studied UV mutagenesis in normal human cells as well as cells derived from xeroderma pigmentosum (XP) patients (group A) whose cells have a profound defect in DNA repair and are almost totally unable to excise UV photoproducts and who are extremely sensitive to sunlight-induced skin cancer (Robbins et al., 1974). The point mutation frequency increased up to 100-fold with UV dose. Mutations were infrequent at potential TT dimer sites. Ninety-three precent of the mutant plasmids from XP cells showed G·C → T·A transition compared to 73% in the normal cells. A

significantly lower frequency of transversion mutations was observed with the XP cells (6%), compared to the normal cells (25%). These investigators concluded that the G·C → A·T transition (at sites other than TT dimer sites) present in the XP spectrum may, therefore, be of greater importance in UV-induced cutaneous carcinogenesis than are the transversion and multiple base substitution mutations.

In the case of UV-irradiation, the high frequency of G·C → A·T transition in the normal human cells was similar to that seen in CV1 cells after *in vitro* plasmid treatment with UV (Hauser *et at.*, 1986). It was also similar to that observed using the 293 cells-*lacI* shuttle system (Lebkowski *et al.*, 1985) and in the mouse cells-lambda phage integrated system (Glazer *et al.*, 1986). It has been suggested that the lesions that initiate the G·C → A·T transition are dipyridine photoproducts in which as A is inserted opposite a C by the polymerase (Bredberg *et al.*, 1986; Hauser *et al.*, 1986).

5. Stably Replicating Shuttle Vector Systems

Another appoach to solve the problem of the high spontaneous mutation frequency of transfected shuttle vectors in mammalian cells and the inefficient replication of some SV40 shuttle vectors is to use stably replicating shuttle vector system. If most of the mutations incurred by transiently replicating vectors are early events targeted to intracellular damage during the transfection process, then stably replicating vectors could offer a solution to the problem. The initital establishment of stable vectors via transfection would be expected to lead to some mutation of the incoming DNA. However, since this mutation frequency is about 1% - 0.01%, it should be relatively

easy to clone cell lines which carry only wild-type vector DNA. If no appreciable mutagenesis occurs during subsequent vector replication, the mutation frequency of vector DNA purified from such cell lines should remain low. In other words, stable vector system potentially allow one to clone cells containing vector molecules which did not suffer any damage to their DNA during the process of transfection. This procedure is not possible in cells transfected with transiently replicating vectors because vector replication prevents long-term survival of these cells.

In contrast to transiently replicating vectors, stably replicating vectors replicate in the nuclei of permissive cells in synchrony with DNA systhesis period of the cell cycle of the transfected cells. They generally attain a fairly stable copy number per cell, but the number is very much lower than that achieved by transiently replicating vectors. Immortalized cell lines transfected with stably replicating vectors are generally able to carry the vectors as extrachomosomal DNA indefinitely. Examples of stable replicating vectors are those derived from the bovine papillomavirus (BPV) and the human herpesvirus Epstein-Barr (EBV).

Unlike vectors based on EBV, vectors based on BPV show high mutation frequency, even in clonal lines (Ashman and Davidson, 1985). The mutations, mainly rearrangements, may due to the inherent instability of BPV vectors (Dimaio et al., 1982; Schenborn et al., 1985). Therefore, BPV-based vectors were found to be unsuitable for mutagenesis studies.

Autonomously replicating vectors have been derived from EBV by Sugden and colleagues (Yates et al., 1984; 1985; Sudgen et al., 1985).

These vectors contain a *cis*-acting element (*oriP*, the origin of replication) of EBV that permits replication and stable maintenance of recombinant plasmids in the nuclei of permisive cells; the *trans*-acting gene coding for the EBV nuclear antigen 1 (EBNA-1) that is required for activation of the origin; a gene coding for hygromycin resistance for selsction in mammalian cells; and portions of plasmid pBR322 for selection and replication of the shuttle vector in bacteria. Sudgen *et al*. (1985) showed that these vectors replicate stably at copy numbers of 5-100 per cell in wide variety of human and other primate cell lines as long as hygromycin in maintained. The high efficiency with which EBV-derived vectors can be introduced, selected and maintained as plasmids in a variety of mammalian cells indicated that they can provide a powerful tool for mutagenesis studies in mammalian cells.

Drinkwater and Klinedinst (1986) have constructed a shuttle vector based on the EBV-derived vector of Sugden *et al*.(1985) for studying mutagenesis in EBV-transformed human lymphoblastoid cell lines. Their vector also contains the 1.1kb HSV *tk* gene as the target for mutagenesis. This vector originally did not contain the EBNA-1 gene so that it could replicate only in cells containing the EBNA-1 antigen. After introduction of this vector into an EBV-transformed human lymphoblastoid cell line by electroporation, approximately 2% of the transfected cells became hygromycin resistant. Plasmid DNA isolated from cells which had been grown in the presence of hygromycin for ten population doublings posttransfection contained mutations in the target HSV *tk* gene at a frequency of 6×10^{-5}. This frequency was 4-fold higher than that observed for the vector which only replicated in *E. coli*. However, it is 2-fold lower than the background frequency

obtained in the best transiently replicating system.

These plasmid-containing cells were treated with N-ethyl-N-nitrosourea (ENU). The frequency of mutations in the HSV tk gene increased up to 15-fold with 1mM ENU. Drinkwater and Klinedinst (1986) showed that the induction of mutations by ENU in the plasmid-encoded HSV tk gene and in the cellular gene for HPRT follows a similar dose-response. Characterization of HSV tk by restiction mapping showed that 30% of the induced mutants from populations of cells contained detectable deletions (>30bp). This result indicates that ENU is a principal base substitution mutagen. The proportion of deletions was large than would be expected. This may indicate that vector containing deletion mutations via the transfection process still existed in the transfected populations. The problem could be avoided by using cloned plasmid-containing cell lines which have a very low background of deletions as target for studies of induced mutagenesis.

Calos and coworkers (DuBridge et al., 1987) contructed other EBV-derived vectors containing the oriP, the gene coding for EBNA-1, the gene for hygromycin resistance, and sequences of pBR322 for selection and replication in bacteria as does those in vectors of Sugden et al. (1985). Their vectors also contain the lacI gene as the target for mutation, and the SV40 origin of replication. These vectors were transfected into 293 cells and hygromycin resistant colonies were selected. These EBV vectors carrying the bacterial lacI gene were than established in 293 cells and later returned to E. coli for rapid detection and analysis of lacI mutations. The background frequency of lacI mutants derived from vectors that had stably maintained in the populations of 293 cells was 2-5 x 10^{-4}. These values are comparable

to mutant frequency of 4 x 10^{-4} obtained with a SV40 vector transiently replicating in 293 cells.

In contrast, the majority (16/18) of clonal cell lines created by establishment of the lacI-EBV vector show spontaneous mutant frequency of less than 10^{-5} and therefore are suitable for studies of induced mutation. Their results also indicate that the high mutation frequency which is seen generally in transiently replicating plasmids, and in pooled populations or sporadic isolated clones with stably replicating vectors, is the result of transfection, not replication.

A cloned vector-containing cell line which carries approximately ten copies of EBV-lacI vectors and has a spontaneous background of 6.4 x 10^{-6} was treated with 1mM of N-methyl-N-nitrourea (MNU) (DuBridge et al., 1987). Vector DNA was harvested and assayed for lacI mutations. The observed induced mutation frequency of 2.1 x 10^{-3} is more than 300-fold above the background frequency. The MNU-induced lacI mutations were subjected to genetic analysis. They found 34 independent nonsense mutations among the 225 lacI mutations (15%).

Of the 34 nonsense mutations, 33 resulted from G·C → A·T transition, the other one being G·C → T·A transversion. These investigators also sequenced 27 of the nonsuppressible mutations induced by MNU. Of the 27 mutations, 26 represent G·C → A·T transition, the single exception being an A·T → G·C transition. The mutagenic lesion for G·C → A·T transition is presumably addition of the alkyl group to the O^6 position of guanine, leading to mispairing with thymine at the first round replication and fixtion of the full transition at the next round (Drake and Baltz, 1976). The alkylations of the O^4 position of thymine could also potentially cause mispairing,

leading to A·T → G·C transition (Singer et al., 1986).

These studies have also provided support for the mutational theory of cancer in connection with the experiments of Barbacid and his colleagues (Sukumar et al., 1983; Zarbl et al., 1985). In those studies, MNU was used to induce mammary from most of the animals contained activated Ha-ras genes. In 48 out of 48 cases, the altered ras genes contained a G·C → A·T transition in the second base of the code for amino acid 12.

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CHAPTER II

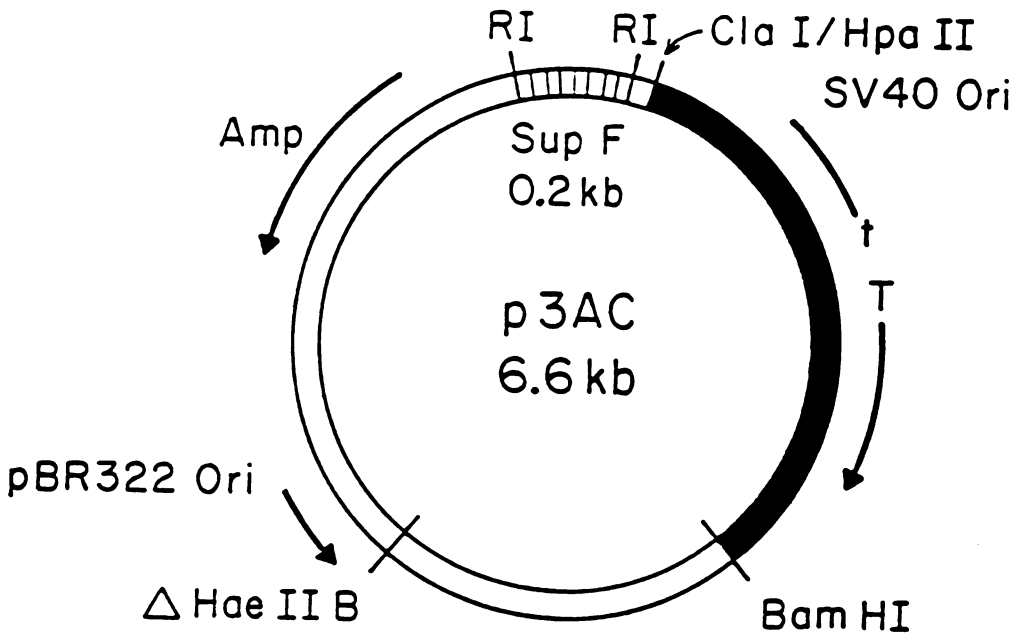
Establishment of a Suitable Shuttle Vector System to Study Molecular Mutagenesis

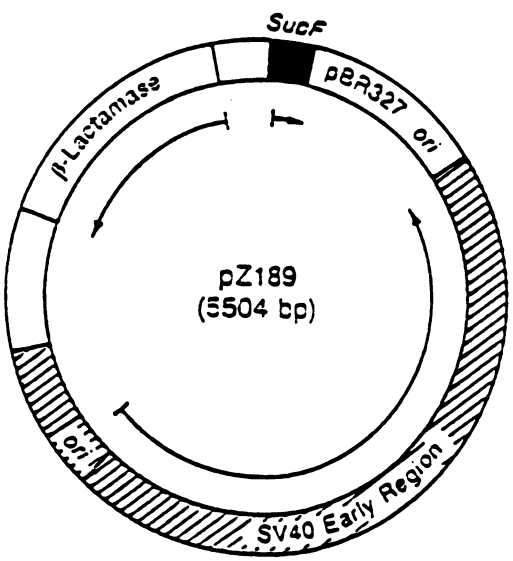
A. The Shuttle Vector Mutagenesis System

Two shuttle vectors, p3AC and pZ189, were used in my studies for the thesis. Vector p3AC was constructed and supplied by Summers and his colleagues (Sarkar et al., 1984); pZ189 was constructed by Seidman and his colleagues (Seidman et al., 1985) and supplied to me by Dr. Kenneth Kraemer of the National Cancer Institution. The structure of the vectors is shown in Figure 1. Both vectors contain the origin of replication and large T-antigen gene from SV40 virus which allows them to replicate in mammalian cells, the gene coding for ampicillin resistance, the bacterial origin of replication from pBR322 or pBR327 plasmid, and the same target gene, supF, coding for a tyrosine supressor tRNA. The major difference between these two vectors is that the supF gene in pZ189 is strategically located between two genes essential for recovery of the plasmid in bacteria (the gene for ampicillin resistance and the bacterial origin of replication). Therefore, plasmids which contain large deletions in the supF gene which extend into either of these two essential genes are not recovered for mutant analysis, which lowers the apparent frequency of background mutants.

The mammalian cell line used in my earliest studies was COS7, monkey kidney cells transformed with SV40 virus containing a defective origin. These cells produce high levels of T-antigen, which

Figure 1. Structure of p3AC and pZ189. pZ189 adapted from Hauser et al. (1986), Mol. Cell. Biol. 6: 277-285. Abbreviations: Amp, ampillin resistance gene (β-lactamase); Ori, origin of replication; RI, EcoRI site; t and T, small and large T antigen.

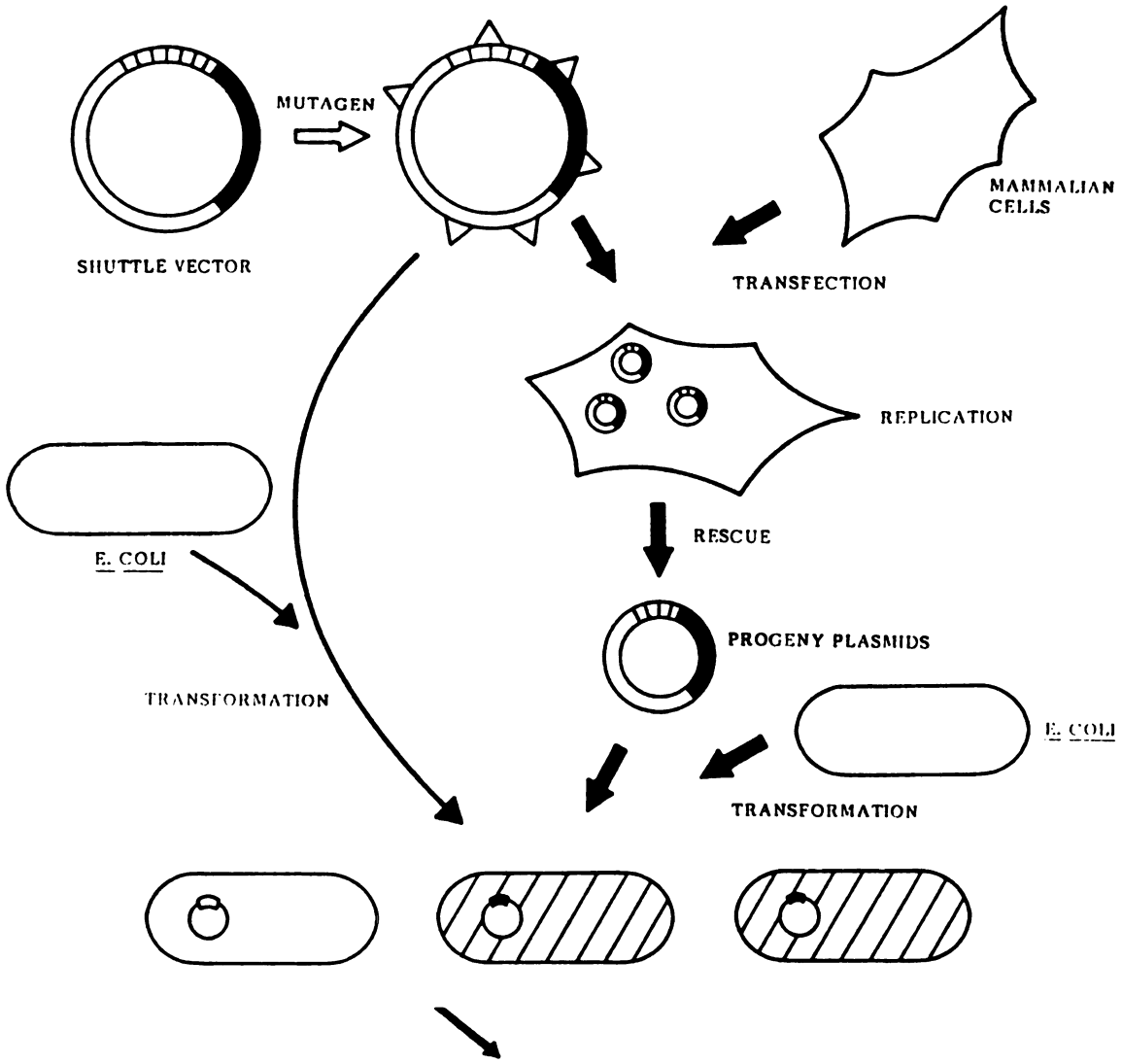

RI
RI
Cla I/Hpa II
SV40 Ori
Amp
Sup F
0.2kb
t
T
p3AC
6.6kb
pBR322 Ori
△ Hae II B
Bam HI


SucF
pBR327 ori
β-Lactamase
pZ189
(5504 bp)
ori
SV40 Early Region

facilitates replication of the SV40-based shuttle vector (Gluzman, 1981). In my later experiments the host cells used were a human cell line, designated 293, which was developed by transforming human embryonic kidney cells with adenovirus 5 DNA fragments (Graham et al., 1977). Substitution of the 293 cell line for COS7 cells as the host cells for my studies was suggested to me by Dr. Calos of Stanford University who observed that shuttle vectors exhibited a lower background in 293 cells than in other host cell lines (Lebkowski et al., 1984). Dr. Calos supplied me with the 293 cells.

Figure 2 diagrams the shuttle vector mutagenesis system I employed. Shuttle vector is exposed to reactive forms of carcinogens in vitro. The carcinogen-treated or untreated plasmid is transfected into mammalian cells using the DNA-calcium-phosphate coprecipitation technique (Graham and Van der Eb, 1973; Chu and Sharp, 1981). The plasmid enter the nucleus of the cells where DNA replication and mutagenesis taken place. 48 hr after DNA transfection, progeny plasmid is extracted from the mammalian cells and separated from large molecular weight cellular DNA by the procedure of Hirt (1967). In order to distinguish independent mutants with identical mutations from putative siblings derived from a single event, progeny plasmid obtained from the cells in each dish is kept separate from the rest and assayed separately. Progeny plasmid is purified by phenol extraction, digested with RNase A, followed by proteinase K, purified again by phenol extraction, precipitated with ethanol, resuspended in buffer, and treated with a restriction enzyme DpnI to destroy any DNA that did not replicate inside the mammalian cells. As shown in Figure 3, if any input plasmid remains unreplicated, it will be destroyed by DpnI

Figure 2. The shuttle vector mutagenesis system. The triangles on the shuttle vector represent carcinogen residues.



ISOLATE MUTANT PLASMIDS FROM WHITE TRANSFORMANTS

↓

CHARACTERIZE MUTANT PLASMIDS BY GEL ELECTROPHORESIS

↓

SEQUENCE PUTATIVE POINT MUTATIONS

↓

ANALYZE THE SPECIFIC LOCATION OF DNA CHANGES

Figure 3. Digestion of DpnI on plasmid with the bacterial methylation pattern. DpnI digests DNA with methylated adenine on the 5′ GATC 3′ sequences. The thicker circles represent plasmids that have replicated in mammalian cells. The thiner circles are the input plasmids that was first prepared from bacteria.

because it has a methylation pattern which differs from that of plasmid which has replicated in mammalian cells. Progeny plasmid is then assayed to see which contains mutations in the supF gene.

To detect supF mutants, the DpnI resistant progeny plasmid is introduced into indicator bacteria by the procedure of Hanahan (1983). The indicator bacteria E. coli SY204 (Sarkar et al., 1984) carries an amber mutation in the β-galactasidase gene so that bacterial transformants containing a plasmid with functioning supF gene which suppresses the amber mutation can form blue clonies on 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) plates containing ampicillin. Bacterial transformants containing plasmid with a mutated, inactive supF gene form white colonies. Only those bacteria that have taken up a plasmid can form colonies on these agar plates because the medium contains ampicillin. The bacteria used for transformation with plasmid are ampicillin sensitive unless they receive a plasmid containing the gene coding for ampicillin.

The white colonies are isolated and the mutant plasmid DNA from each of these colonies is then amplified, extracted, and analyzed by gel electrophoresis for altered DNA mobility (gross alterations). Plasmid with a normal gel pattern are considered to contain putative point mutations in the supF gene and are further characterized by DNA sequencing. Examples of an agarose gel pattern and a DNA sequencing pattern are shown in Figures 4 and 5.

Figure 4. Agarose gel electrophresis analysis of plasmids containing mutations at the supF gene. Plasmid DNA was prepared from overnight bacterial culture and isolated from E. coli by rapid alkli-lysis procedure. Plasmid DNA was applied and run on 0.8% agarose gel. The gel was stained with ethidium bromide (0.5 µg/ml) and the gel was photographed using transmitted UV light. Lane Z shows wild-type pZ189. Lanes 2, 3-8, 10, 11 show supF mutants without altered gel mobility. Lane 1 shows a supF mutant with gross deletions. Lane 9 shows a supF mutant with gross deletions. Lane M shows HindIII-digested lambda DNA as molecular weight markers.

1 2 Z M 3 4 5 6 7 8 9 10 11

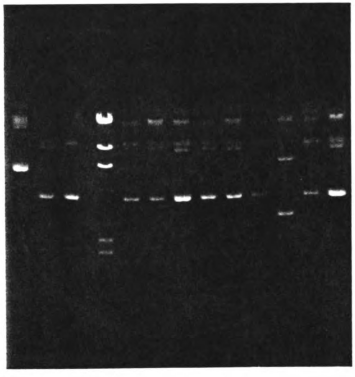
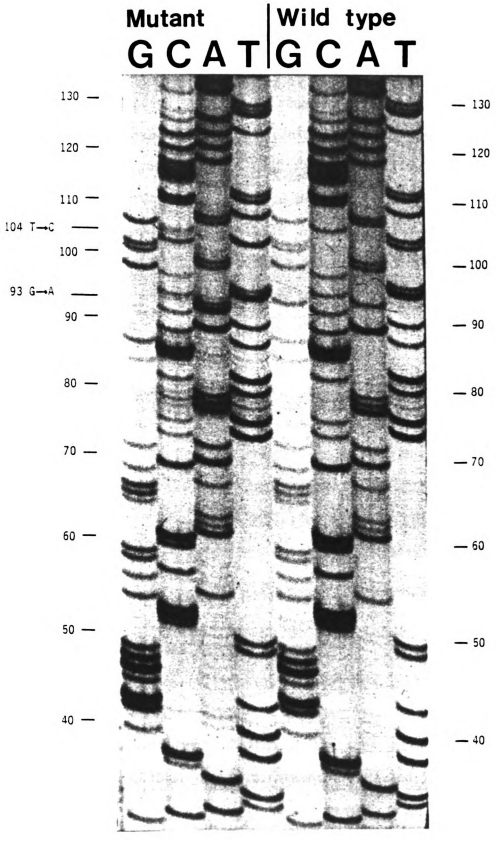


Figure 5. DNA sequencing analysis of a plasmid containing mutations in the supF gene. Part of the sequence of a supF mutant and of the wild type DNA showing the 93 G → A and the 104 T → C.


Mutant
Wild type
G C A T G C A T
130
120
110
104 T→C
100
93 G→A
90
80
70
60
50
40

B. Determination of Experimental Conditions to Maximize the Yields of Progeny Plasmid from Mammalian Cells.

It is critical to rescue a sufficient amount of the progeny plasmid from mammalian cells to be able to analyze the frequency of mutants and have enough mutants to sequence. At the begining of my studies, I determined the optimal yield of the progeny plasmids by transfecting 0.1 μg to 20 μg of p3AC DNA into ~10 x 10^6 COS7 cells, using the $CaPO_4$ coprecipitation method of Chu and Sharp (1981). After the plasmid was rescued, the yield of progeny plasmid was analyzed by Southern blot analysis (Southern, 1975) (Figure 6). The results showed that approximately the same yield of progeny plasmid was generated using 1 to 20 μg of plasmid for transfection. Therefore, 1 μg of p3AC was used for further experiments.

To further optimize the yield of progeny plasmid, a time course experiment was conducted. One ug of plasmid was transferred into ~10 x 10^6 COS cells, and the progeny plasmid was harvested at 45 hr, 49hr, and 57 hr after transfection. The Southern blot analysis (Figure 7) showed that the yield of plasmid harvested from COS cells at 49 hr was increased over the yield at 45 hr, but the yeild after 57 hr was not further increased.

C. Reducing the Background Mutant Frequency.

In the study of p3AC replicating in COS7 cells, the DpnI resistant progeny plasmid obtained from more than ten COS7 transfection experiments was assayed for mutations in the supF gene. Among a total number of 21,300 bacterial transformants analyzed, 77 were supF mutants. Therefore, the mutant frequency was 36 x 10^{-4}. This mutant

Figure 6. The relationship between the yield of p3AC generated during replication in COS7 cells and the amount of input plasmid. Various amount of p3AC were transfected into COS7 cells as indicated above lane 1-6. 1/20th of the total volume of each DNA sample extracted from COS7 cells was digested with DpnI and EcoRI (lane 1-6) and applied to the gel. Blot hybridization analysis was carried out by the procedure of Southern (1975), but the probe was S^{35}-nick translated p3AC. Lanes 8-10 represent 0.1 ng, 1 ng, 10 ng of EcoRI-digested input p3AC used as markers, respectively. Lane 7 contains 10 ng of DpnI-digest p3AC. This enzyme is used to distinguish the plasmids replicated in E. coli from those replicated in COS7 cells.

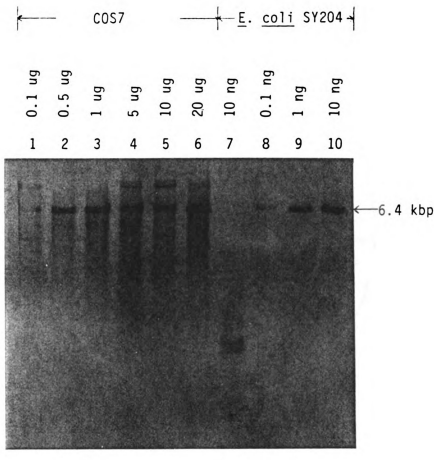
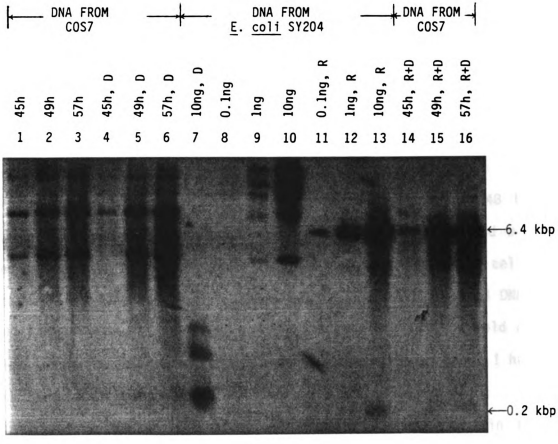

COS7
E. coli SY204
0.1 ug
0.5 ug
1 ug
5 ug
10 ug
20 ug
10 ng
0.1 ng
1 ng
10 ng
1 2 3 4 5 6 7 8 9 10
6.4 kbp

Figure 7. Time course experiment of replication of p3AC in COS7 cells. 1/20th of the total volume of each DNA sample extracted from COS7 cells at various times (as indicated above lanes 1-6 and lanes 14-16) was digested with DpnI (lanes 4-6) or with DpnI plus EcoRI (lanes 14-16), or was not digested with enzymes (lanes 1-3). Various amounts of input p3AC were used as markers (the amounts are indicated above lanes 7-13). Lanes 8-10 shows that p3AC was not digested by enzymes . Lanes 11-13 shows that p3AC was digested with EcoRI. Lane 7 shows that p3AC was digested with DpnI. Blot hybridization analysis was carried out by the method of Southern (1975). The probe was ^{32}P-nick translated p3AC.


DNA FROM COS7
DNA FROM E. coli SY204
DNA FROM COS7
45h
49h
57h
45h, D
49h, D
57h, D
10ng, D
0.1ng
1ng
10ng
0.1ng, R
1ng, R
10ng, R
45h, R+D
49h, R+D
57h, R+D
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
6.4 kbp
0.2 kbp

frequency was somewhat lower than that obtained in the earliest shuttle vector systems developed, but higher than the frequency reported by Sarkar et al. (1984) (i.e. 23 x 10^{-4}). It proved to be too high to observe an increase in mutant frequency induced by carcinogens (see Chapter III). At the conclusion of my study of the frequency and kinds of mutants induced when BPDE-treated plasmid (p3AC) replicated in COS7 cells, I tried to find out if I could lower the mutant frequency, as well as increase the yield of plasmid, by transfecting a variety of mammalian cell lines, including SV40-transformed normal human cells (GM637), SV40 transformed xeroderma pigmentosum cells (XP12RO), and an human embryonic kidney cell line, 293 transformed with the adenovirus-5 DNA fragments.

Plasmid p3AC was transferred into these cell lines and 48 hr later, progeny plasmid was rescued from the cells and the yield was assayed by Southern blot analysis. The results showed that GM637 cells contained an abundant amount of autonomously replicated SV40 viral DNA, but little p3AC DNA. The XP12RO cells also produced little yield of plasmid. Only the 293 cells produced yields comparable to those I had obtained from COS7 cells.

The second strategy to lower the background frequency in my studies was to use another vector, pZ189, in which the target gene is placed between the gene for ampicillin resistance and the origin for replication in E coli. Tests with that plasmid showed a low background frequency of mutants, as well as a reduced recovery of spontaneous gross deletions produced during replication in monkey CV1 cell line (Seidman et al., 1985; Hauser et al., 1986). I then applied this vector in the mutagenesis assay, and determined the background

mutant frequency as well as the yield of progeny plasmid when pZ189 replicated in either COS7 or 293 cells. The results were compared with what I had found with p3AC.

Comparison of the frequency of spontaneous supF mutants produced during replication of these two plasmids in COS7 cells is shown in Table 1. The spontaneous mutant frequency of vector pZ189 was 3.6 fold lower than that of vector p3AC. The yield of progeny plasmids of pZ189 recovered from COS7 cells was approximately ten fold higher than that of p3AC (data not shown). These data indicate that pZ189 is better for investigating mutations in induced in the supF gene during replication in mammalian cells than is p3AC.

I also compared the yield of the two plasmid in 293 cells. A very poor recovery yield of p3AC progeny plasmids during replication in 293 cells was obtained. In contrast, the yield of pZ189 progeny plasmids during replication in 293 was very high. The yield of pZ189 progeny plasmids was five times higher than that obtained in COS7 cells and the background mutant frequency (1×10^{-4}, Table 2) was ten times lower than that observed in COS7 cells (Table 3). Agarose gel analysis of mutants derived from pZ189 showed that gross deletion/insertion mutations were greatly reduced; only 16% compared to 78% derived from p3AC replication in COS7 cells (see Chapter III and IV). Therefore, 293 cells proved to be particularly good for the pZ189 shuttle vector mutagenesis studies.

Table 1. Comparison of the frequency of spontaneous *supF* mutants generated during replication of p3AC or pZ189 in COS7 cells.

Vector	Number of transformants	Number of *supF* mutants	Number of *supF* mutants x 10^4
p3AC	21,300	77	36
pZ189	34,710	36	10

Table 2. Frequency of *supF* mutants generated during replication of untreated pZ189 in COS7 cells.

Transfection experiment number	Number of transformants	Number of *supF* mutants
1	15,470	14
2	1,190	0
3	4,090	2
4	4,210	7
5	5,325	7
6	1,885	2
7	2,540	4
Total	34,710	36

Average frequency = 10.3×10^{-4}

Table 3. Frequency of spontaneous *supF* mutants observed during replication of pZ189 in 293 cells.

Transfection experiment number	Number of transformants	Number of *supF* mutants	Frequency of *supF* mutants x 10^4
1	68,435	4	0.58
2	25,410	2	0.79
3	10,425	1	0.96
4	3,375	0	—
5	3,235	0	—
6	13,560	2	1.50
7	10,590	1	0.94
8	11,310	3	2.65
9	2,735	0	—
10	5,455	1	1.80
Total	154,530	14	0.91

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CHAPTER III

Kinds of Mutations Formed When a Shuttle Vector Containing Adducts of Benzo[a]pyrene-7,8-diol-9,10-epoxide Replicates in COS7 Cells

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SUMMARY

We have investigated the kinds of mutations induced when a shuttle vector containing covalently bound residues of the (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) replicates in the monkey kidney cell line COS7. The target for detecting mutations was the 200-base pair gene for a tyrosine suppressor tRNA (supF), inserted at the EcoRI site in shuttle vector p3AC (Sarkar et al., Mol. Cell Biol. 4:2227-2230, 1984). When introduced by transformation, a functioning supF gene in progeny plasmid recovered from COS7 cells allows suppression of a lacZ amber mutation in the indicator Escherichia coli host. Treatment of p3AC with BPDE caused a linear increase in the number of BPDE residues bound per plasmid. Untreated plasmids and plasmids containing 6.6 BPDE residues were transfected into COS7 cells, and the progeny were assayed for mutations in the supF gene. The frequency of mutants generated during replication of the BPDE-treated plasmids was not higher than that from untreated plasmids, but the two populations differed markedly in the kinds of mutations they contained. Gel electrophoresis analysis of the size alterations of 77 mutant plasmids obtained with untreated DNA and 45 obtained with BPDE-treated DNA showed that the majority of the mutant progeny of untreated plasmids exhibited gross alterations, principally large deletions. In contrast, the majority of the mutants generated during replication of the BPDE-treated plasmids contained only minor alterations, principally point mutations. Sequence analysis of progeny of untreated plasmids containing putative point mutations showed insertions and deletions of bases and a broad spectrum of base

substitutions; in those from BPDE-treated plasmids, all base substitutions involved guanosine·cystosine pairs.

INTRODUCTION

As part of a study of the mechanisms of carcinogenesis, we are investigating at the sequence level the specific kinds of mutations induced in mammalian cells by carcinogens, including (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE), a major reactive metabolite of the widely distributed environmental carcinogen benzo[a]pyrene (26). Several investigators have examined the kinds of mutations induced by BPDE in bacteria, but not in mammalian cells. For example, Eisenstadt et al. (8) treated nucleotide excision repair-deficient Escherichia coli with BPDE and genetically analyzed a large number of nonsense mutations in the lacI gene for the kinds of base substitutions induced. The majority proved to be G·C→T·A. Chakrabarti et al. (6) treated plasmid with BPDE, isolated a specific fragment in the gene for tetracycline resistance, ligated it back into the complementary part of unmodified plasmids, and then transformed E. coli with the chimeric plasmid containing the localized patch of BPDE adducts to determine their effect on plasmid survival and mutagenesis. They found that the majority of the mutations did not involve major alterations in the target gene. Sequence analysis of five mutants showed that three had G·C pair deletions, one had a G·C→T·A transversion, and one had a A·T → G·C transition. Mizusawa et al. (18) conducted a similar type of study, treating plasmids with BPDE and transfecting them into various E. coli strains with different repair capacities to study the effects of BPDE on plasmid survival in the various recipients and on the frequency of mutants (galK$^+$ → galK). Sequence analysis was not carried out because of the large size of the

gene, but gel electrophoresis analysis of the mutant plasmids taken from the bacteria showed that the majority (15 of 16) did not involve detectable alterations in the size of the DNA fragment of interest. In a related study involving a *galK* → *galK*$^+$ selection system and a 230-base pair (bp) transcription termination sequence as the target for mutations, sequence analysis of the DNA from 15 *galK*$^+$ colonies revealed three with mutations in the region of interest (19). Two of these showed insertion of T·A into a cluster of T·A base pairs; the third showed a deletion of G·C from a cluster of G·C base pairs.

Indirect evidence suggests that the mechanisms of mutagenesis by certain carcinogens in mammalian cells can differ from that in bacteria (2). Therefore, we treated a shuttle vector with tritium-labeled BPDE and transfected it into the monkey kidney cell line COS7 to allow replication to occur. Mutant progeny plasmids were identified by transforming an *E. coli* indicator host and analyzed for mutations in the target gene, the 200-bp tyrosine suppressor tRNA (*supF*). We found that the frequency of gross alterations formed in the progeny of BPDE-treated plasmid was significantly lower than with untreated plasmid, and DNA sequence analysis showed that, unlike the case for the untreated plasmid, the majority of the point mutations obtained with BPDE-treated plasmid were base substitutions, and all of these involved a G·C base pair transition or transversion.

MATERIALS, METHODS, AND RESULTS

Cells and plasmids. The 6.6 kbp shuttle vector used p3AC, constructed by Sarkar *et al.* (23) and provided by W.C. Summers, contains parts of a pBR322 plasmid including the origin of replication and the gene for ampicillin resistance (*amp*), the *Bam*HI and *Hpa*II fragments of the early region of simian virus 40 DNA, and the 200-bp *supF* gene, which serves as the target gene for mutagenesis and subsequent sequencing. The ampicillin-sensitive indicator bacterial host, *E. coli* SY204, carries an amber mutation in the β-galactasidase gene (23). The eucaryotic host of the shuttle vector, COS7 simian cells (10), were grown in Eagle's minimal essential supplemented with 0.2mM L-serine, 0.2mM L-aspartic acid, 1mM sodium pyruvate and 10% fetal calf serum (Gibco Laboratories, Grand Island, NY).

Preparation of plasmid DNA containing BPDE adducts. Plasmid were prepared by an alkaline lysis procedure (16) and purified by ethidium bromide-CsCl density centifugation. A small volume (1-16 µl) of generally tritiated BPDE (692 mCi/mmole, 0.3 mg/ml in tetrahydrofuran, 96% pure [Midwest Research Institute, Kansas City, MO.]), was diluted in anhydrous acetone immediately before use and added to 200 µl of a 0.5-mg/ml solution of DNA in 10 mM Tris hydrochloride-1 mM EDTA buffer, pH 8.0. The mixture, protected from light, was incubated at room temperature for 2 h. Unbound BPDE was removed by three successive ethanol precipitations, and the moles of BPDE residues bound per mole of p3AC was calculated from the A_{260} profile of the DNA and the specific activity. The number of BPDE adducts per molecule of plasmid was proportional to the concentration of BPDE, with 5 µM giving 6.6

BPDE residues per plasmid.

Transfection of COS7 cells and assay of progeny plasmid for supF mutations. COS7 cells in suspension were transfected with untreated plasmid or plasmid containing 6.6 BPDE, using the DNA-calcium phosphate coprecipitation method of Chu and Sharp (7). After 48 h, plasmid DNA was extracted by the procedure of Hirt (13), purified with phenol, treated with RNase A (50 μg/ml) at 37°C for 1 h, followed by proteinase K (100 μg/ml) at 50° C for 2 h, and then extracted with phenol-chloroform, precipitated with ethanol, and further purified by drop dialysis (26). The purified plasmid was treated with DpnI to digest any input plasmid and then was used to transform SY204 bacterial cells to ampicillin resistance, using the method of Hanahan (11). Transformants were selected on Luria-Bertani (LB) broth plates containing 5-bromo-4-chloro-3-indolyl- β-D-galactoside (X-Gal) (40 mg/liter), an inducer, isopropyl- β-D-thiogalactoside (20 mg/liter), and ampicillin (50 mg/liter). Cells containing plasmids with a functioning supF gene which suppresses the amber mutation can form blue colonies on X-Gal plates, whereas cells containing plasmids with a mutated, inactive supF gene form white colonies. The frequency of mutants was determined by dividing the number of white colonies by the total number of colonies. The results are shown in Table 1.

Charaterization of mutant plasmids by gel electrophoresis and sequencing. Bacterial cells from white colonies were restreaked on fresh X-Gal plates containing isopropyl- β-D-thiogalactoside and ampicillin to confirm their phenotype, and their DNA was extracted, purified, and analyzed by electrophoresis on 0.8% agarose gels for altered DNA mobility (gross alterations). Plasmids with normal agarose

Table 1. Frequency of *supF* mutants obtained with BPDE-treated or untreated p3AC that had replicated in COS7 cells.

Treatment	No. of transformants	No. of mutants	Mutant frequency
None	21,300	77	36×10^{-4}
BPDE	13,500	45	33×10^{-4}

gel patterns were digested with EcoRl and analyzed by electrophoresis on 6% polyacrylamide gels for changes in the size of the supF gene. The data for 77 mutants from untreated p3AC and the 45 mutants obtained from the progeny of BPDE-treated plasmid are summarized in Table 2. The majority of the mutants obtained after transfection of COS7 cells with untreated p3AC exhibited gross rearrangements, predominantly deletions but also large insertions. In contrast, the majority of the mutant plasmids obtained with BPDE-treated DNA exhibited only minor alterations, and most of these were putative point mutations since they did not show any visible alteration in size on agarose or polyacrylamide gels.

Plasmids with a normal polyacrylamide gel pattern were considered to contain putative point mutations in the supF gene. Seven unambiguously independent mutants of this type derived from untreated plasmids and nine derived from BPDE-treated plasmids were sequenced, using a modification of the dideoxyribonucleotide method of Sanger et al. (22). DNA was prepared and purified through CsCl gradients as described above and denatured by alkali to generate single-strand DNA templates as described previously (28). Polymerization from a pBR322 EcoRI site primer was carried out with the Klenow fragment of DNA polymerase I. [^{35}S] α-dATP (034S; New England Nuclear, Boston, Mass.) and buffer gradient-denatured polyacrylamide gels were used for greater resolution (3). The results are shown in Table 3 and Fig. 1. Among the untreated controls, three of the seven mutants showed a single base substitution G $\rightarrow$ A, G $\rightarrow$ T, and C $\rightarrow$ T; two had two base substitutions; and two had several changes, including single base insertions and deletions. In contrast, five of the nine mutants from

Table 2. Characterization of the kinds of mutations generated in p3AC during replication in COS7 cells.

Characterization of mutant p3AC vectors	No. (%) of mutants	
	No-treatment (controls)	BPDE-treated (6.6 adducts/p3AC)
MINOR ALTERATION		
Putative point mutations	12 (15.6%)	15 (33.3%)
Small deletions or insertions (delete or insert <30bp)	5 (6.5%)	8 (17.8%)
GROSS ALTERATION		
Deletions (delete ~200bp)	25 (32.5%)	12 (26.7%)
Large deletions (delete >1 kb[a])	24 (31.1%)	4 (8.9%)
Large insertions (insert >1 kb)	11 (14.3%)	6 (13.3%)
TOTAL MUTANTS	77 (100%)	45 (100%)

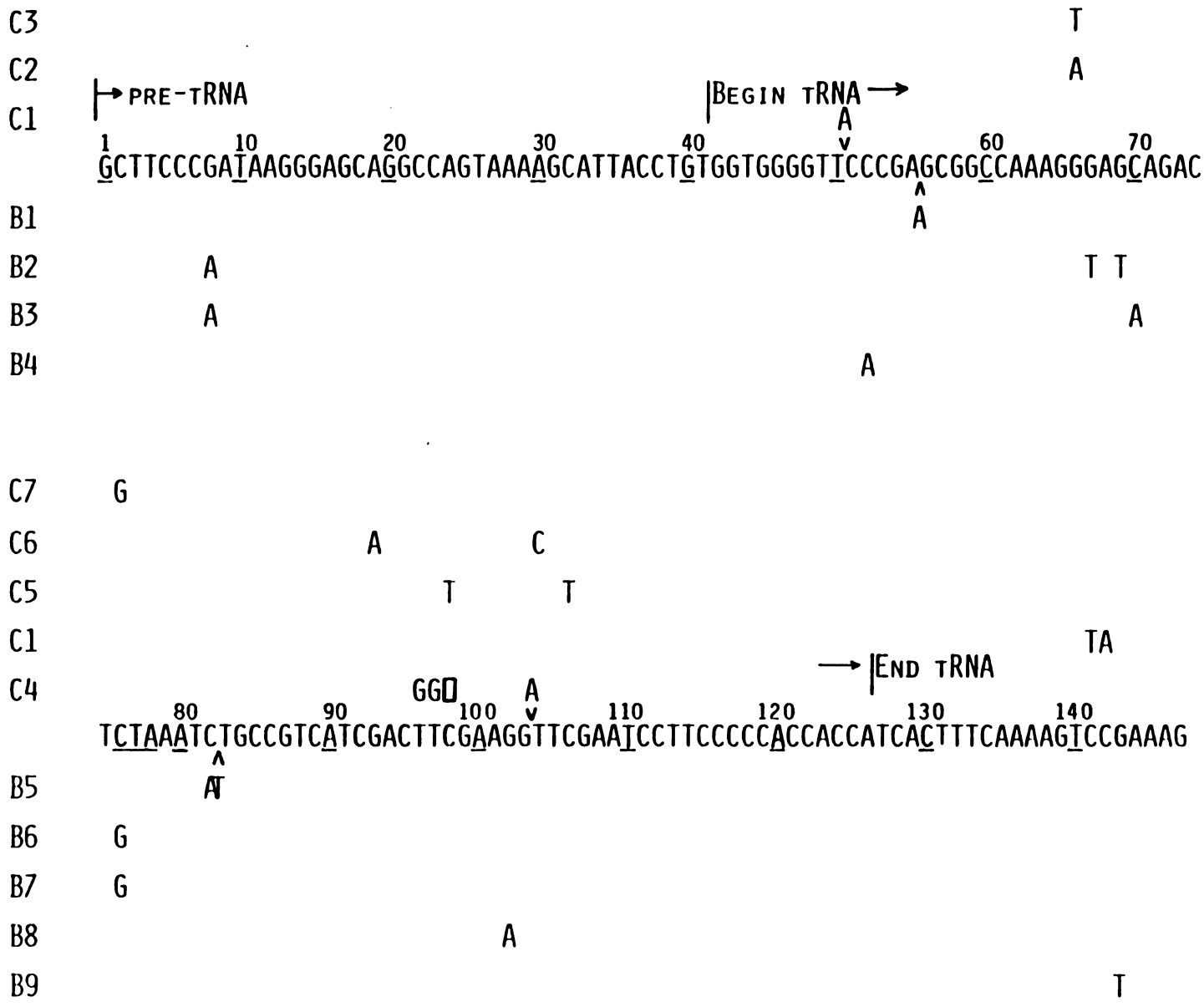
[a]kb, Kilobase

Table 3. Analysis of the kinds of base changes found in the *supF* gene of the mutant p3ACs analyzed[a].

Type of change	Untreated p3AC		BPDE-treated p3AC	
	Times occurring	% of total	Times occurring	% of total
G·C → T·A	2	14	6	46
G·C → A·T	5	36	3	23
C·G → G·C	1	7	2	15
A·T → G·C	1	7	0	0
A·T → C·G	2	14	0	0
Insert an A	2	14	1	8
Insert a T	0	0	1	8
Delete a C	1	7	0	0

[a]Only those p3ACs containing putative point mutations were analyzed at the sequence level.

Figure 1. Distribution of mutants in the supF gene of p3AC. The DNA strand shown is the strand synthesized during the DNA sequencing reaction, using the EcoR1 rightward-sequencing primer. It corresponds to the tRNA sequence. C1-C7 refers to mutants obtained with untreated plasmids; B1-B9 refers to those from treated plasmids.


C3
C2
C1
PRE-tRNA
BEGIN tRNA
1 10 20 30 40 60 70
GCTTCCCGATAAGGGAGCAGGCCAGTAAAAGCATTACCTGTGGTGGGGTICCCGAGCGGCCAAAGGGAGCAGAC
B1
B2
B3
B4
C7
C6
C5
C1
C4
END tRNA
80 90 100 110 120 130 140
TCTAAATCTGCCGTCATCGACTTCGAAGGTTCGAAICCTTCCCCCACCACCATCACTTTCAAAAGICCGAAAG
B5
B6
B7
B8
B9

BPDE-treated plasmids showed a single base substitution; three of nine had two base substitutions; and one had three base substitutions. All of these substitutions involved G·C base pairs. One mutant had a base substitution, but also the insertion of a nucleotide; one had just the insertion of a nucleotide. The location of each change in the gene can be seen Fig. 1.

DISCUSSION

For comparative purposes, it is useful to calculate the mutant analysis data of Table 1 and 2 on the basis of the frequency of each type of mutation. This calculation shows that the frequency of mutant progeny from untreated plasmids carrying minor alterations was 8 x 10^{-4}, but for the BPDE-treated plasmids it was 17 x 10^{-4}. This indicated that BPDE induces point mutations, which supports the findings of King and Brookes (14), who studied the kinds of mutations induced in V79 Chinese hamster cells as detected by DNA hybridization, and those of Aust et al. (2), who showed in human diploid fibroblasts that BPDE does not cause the kinds of mutations (deletions, rearrangements) expected to completely inactivate the gene coding for elongation factor 2, which is involved in diphtheria toxin resistance. That all base substitutions observed with progeny of BPDE-treated plasmid involved G·C pairs is in keeping with the hypothesis that BPDE adducts were involved since this carcinogen predominantly binds to guanine (26,27).

A similar analysis of the data in Tables 1 and 2 for the frequency of mutants carrying gross alterations shows that for the progeny of untreated plasmid this value was 28 x 10^{-4}, with 23 x 10^{-4} being deletions and 5 x 10^{-4} being insertions. This result is not surprising. Evidence from several groups of investigators indicates that, when simian virus 40-based plasmids are introduced into mammalian cells, they are subjected to strand breaking, deletions, duplications, recombination, and more complex rearrangements, including the insertion of sequences from the mammalian cell genome (1,4,5,15,17,20,21).

However, with the progeny of BPDE-treated plasmids, the frequency of mutants carrying such gross alterations was significantly lower than background, only 16 x 10^{-4}, with 12 x 10^{-4} being deletions, compared to 23 x 10^{-4} in the control. (The frequency of insertions was about the same in both populations.) The failure to recover the background level of plasmids carrying deletions suggests that the presence of BPDE adducts interferes in some way with the process that produces these gross rearrangements. Another explanation is that BPDE adducts increase size of the deletions so that they extend into the amp gene. Such plasmids would not be recovered in our assay.

Gross rearrangements were rarely seen when we transformed E. coli SY204 directly with BPDE-treated or untreated p3AC without first transfecting COS7 cells (data not shown). This result agrees with the results of Chakrabarti et al. (6) who did not observe rearrangements following transformation of E. coli with plasmid. Neither did Glazer et al. (9) find rearrangements in the supF gene when it was integrated into a mammalian cell chromosome and the host cell was exposed to mutagens.

One important advantage of using the supF gene as the target for mutation studies at the sequence level is its small size. Another is that the tRNA gene is responsive to base substitutions in many different positions (9,12,23). Recently Seidman et al. (24) reported construction of a shuttle vector, pZ189, in which the supF gene is strategically located between the origin of replication of the plasmid in E. coli and the gene for ampicillin resistance so that the possibility of recovering mutants containing these spontaneous gross rearrangements is greatly decreased. This plasmid will make it easier

to study the types of minor alterations induced by various mutagens (12) and we are currently using it to obtain such data for BPDE. Nevertheless, use of p3AC in the present study made it possible for us to detect the effect of BPDE on the processes responsible for generating the spontaneous gross alterations.

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CHAPTER IV

Kinds of Mutations Formed When a Shuttle vector Containing Adducts of (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene Replicates in Human Cells

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SUMMARY

We have investigated the kinds of mutations induced when a shuttle vector containing covalently bound residues of (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) replicates in human cells. A human embryonic kidney cell line, 293, was used as the eucaryotic host. The target gene for mutation analysis, supF, codes for a tyrosine supppressor tRNA and is strategically located between the origin of replication of the plasmid in Escherichia coli and the gene for a selectable marker, so that the possibility of recovering supF mutants containing gross rearrangements is low. The frequency of supF mutants obtained when untreated plasmid replicated in 293 cells was 1.4×10^{-4}. The frequency with BPDE-treated plasmid increased linearly as a function of the number of adducts, with 16 adducts per plasmid giving 38×10^{-4}. Polyacrylamide gel and agarose gel electrophoresis analysis of 137 plasmids with mutations in the supF gene indicated that 70% (21/30) from untreated plasmids contained deletions or insertions or showed altered gel mobility, whereas only 28% (30/107) of those devired from BPDE-treated plasmid contained such alterations. Of the 86 unequivocally independent mutants derived from BPDE-treated plasmids that were analyzed by sequencing, the majority (60/86) exhibited base substitutions. Mutants exhibiting frameshifts (insertions or deletions of one, two, or four base pairs) were also found, but they were a minority (11/86). In the progeny of BPDE-treated plasmids 61/71 base substitutions observed were transversions, with 45/61 G·C → T·A. Examination of the location of BPDE-induced mutations among the 85 base

pairs in the structure of the tRNA revealed that 30% of the base substitutions occurred at two sites and 44% of the rest occurred at five other hot spots. Only 20% of all these base changes involved a site in which a guanine containing a BPDE adduct is predicted to be labile—i.e., a guanine that has a pyrimidine to its 5′ side.

INTRODUCTION

The molecular mechanisms responsible for the induction of mutations in mammalian cells are not well understood. Attempts to deduce the nature of such mechanisms from the products of the mutagenic events have been hampered up until now by an inability to isolate and analyze newly-mutated genes at the sequence level. However, several elegant systems have recently been devised (1-10) that allow rescue from mammalian cells of DNA containing mutations in target genes whose sequences are known. We have adapted one of these systems to investigate the kinds of mutations induced when DNA containing covalently-bound residues of (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE), a major reactive metabolite of the widely distributed environmental carcinogen benzo[a]pyrene (11), replicates in human cells. Our study of BPDE-induced mutation is part of a larger study of the molecular mechanisms of mutagenesis and carcinogenesis in human cells.

We treated a shuttle vector containing a small defined target gene, supF, which codes for a tyrosine suppressor tRNA. Use of this particular gene offers the advantage that base substitutions at any one of a large number of positions among the 85 nucleotides making up the tRNA structure results in a detectable phenotypic change (6,7,10,12). Use of the human cell line 293 as the eukaryotic host for replicating the plasmid offers the advantage of a very low spontaneous background frequency of mutants (13). With this system we have found that BPDE induces point mutations, principally base substitutions, as a linear function of the number of adducts per plasmid. DNA sequence analysis

of the mutations generated in human cells with BPDE-treated plasmids has allowed us to determine the type of structural alterations induced, as well as their specific location in the target gene. Our results indicate that the majority of the base substitutions observed did not occur at sites predicted by Lobanenkov et al. (14) to be particularly subject to depurination—i.e., ones in which the guanine containing a BPDE adduct has a pyrimidine adjacent to it at the 5′ side.

MATERIALS AND METHODS

Cells and Plasmid. A human embryonic kidney cell line, 293, transformed by adenovirus-5 DNA fragments (15) served as the eucaryotic host. It was obtained from Dr. Michele Calos (Stanford University, Stanford, CA). The cells were grown in Eagle's minimal essential medium supplemented with 0.2mM L-serine, 0.2mM L-aspartic acid, 1mM sodium pyruvate and 10% fetal calf serum (GIBCO). The ampicillin sensitive indicator bacterial host, Escherichia coli SY204, carrying an amber mutation in the β-galactasidase gene, was obtained from William C. Summers (Yale University, New Haven, CT) and has been described (3). The 5504-base-pair shuttle vector used, pZ189, constructed by Seidman et al. (4), contains the gene for a tyrosine suppressor tRNA, supF, which is flanked by two genes essential for recovery of the plasmid in E. coli, i.e., the gene for ampicillin resistance and the bacterial origin of replication, as well as the origin of replication and large tumor antigen gene from simian virus 40. It was provided by Kenneth Kraemer (National Cancer Institute).

Formation of BPDE Adducts on the Plasmid. Plasmid was prepared by using an alkaline lysis procedure and purified by ethidium bromide/CsCl density centrifugation (16). A small volume (1-4 µl) of generally tritiated BPDE (692 mCi/mmole, 0.3 mg/ml in tetrahydrofuran, 96% pure; Midwest Research Institute, Kansas City, MO; 1 Ci = 37 GBq) was added to 200 µl of an 0.5 mg/ml solution of DNA in 10 mM Tris-HCl, 1 mM EDTA buffer, pH 8.0. The mixture was protected from light and incubated at room temperature for 2 hr. Unbound BPDE was removed by three successive precipitations with ethanol and the number of moles of

BPDE residues bound per mole of pZ189 was calculated from the UV_{260} absorption profile of the DNA and the specific activity.

Transfection and Rescue of Replicated Plasmid. Human 293 cells ($0.5\text{-}1 \times 10^6$) were plated into a series of 150-mm-diameter dishes. After 24 hr later they were transfected with 6 μg of plasmid by using calcium phosphate coprecipitation but omitting the glycerol shock (17). After 48 hr, low molecular weight DNA (plasmid) was extracted and separated from cellular DNA by the procedure of Hirt (18), purified with buffered phenol, and treated with RNase A (50 μg/ml) at 37°C for 1 hr, followed by proteinase K (100 μg/ml) at 50° C for 2 hr, then extracted with phenol/chloroform, precipitated with ethanol, and further purified by drop dialysis (19). Care was taken to keep the DNA extracted from the target cells in each individual 150-mm dish separate from the next so that we could know if any mutants arose from the same population (putative siblings). Before the plasmid DNA from each Hirt supernatant was used to transform the indicator bacteria, it was treated with DpnI (20 units) to digest any original input plasmid. This restriction enzyme digests any DNA that still has the bacterial methylation pattern generated when the plasmid was first prepared.

Bacterial Transformation and Mutant Identification. *E. coli* SY204 cells were transformed to ampicillin resistance (20) and selected on Luria-Bertani broth agar plates containing 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (40 mg/liter), an inducer, isopropyl β-D-thiogalactoside (IPTG) (20 mg/liter), and ampicillin (50 mg/liter). Cells containing plasmids with a functioning *supF* gene, which suppresses the amber mutation, can form blue colonies on X-Gal plates, whereas cells containing plasmids with a mutated, inactive *supF*

gene form white or light-blue colonies. Each colony was restreaked on fresh plates to confirm the phenotype.

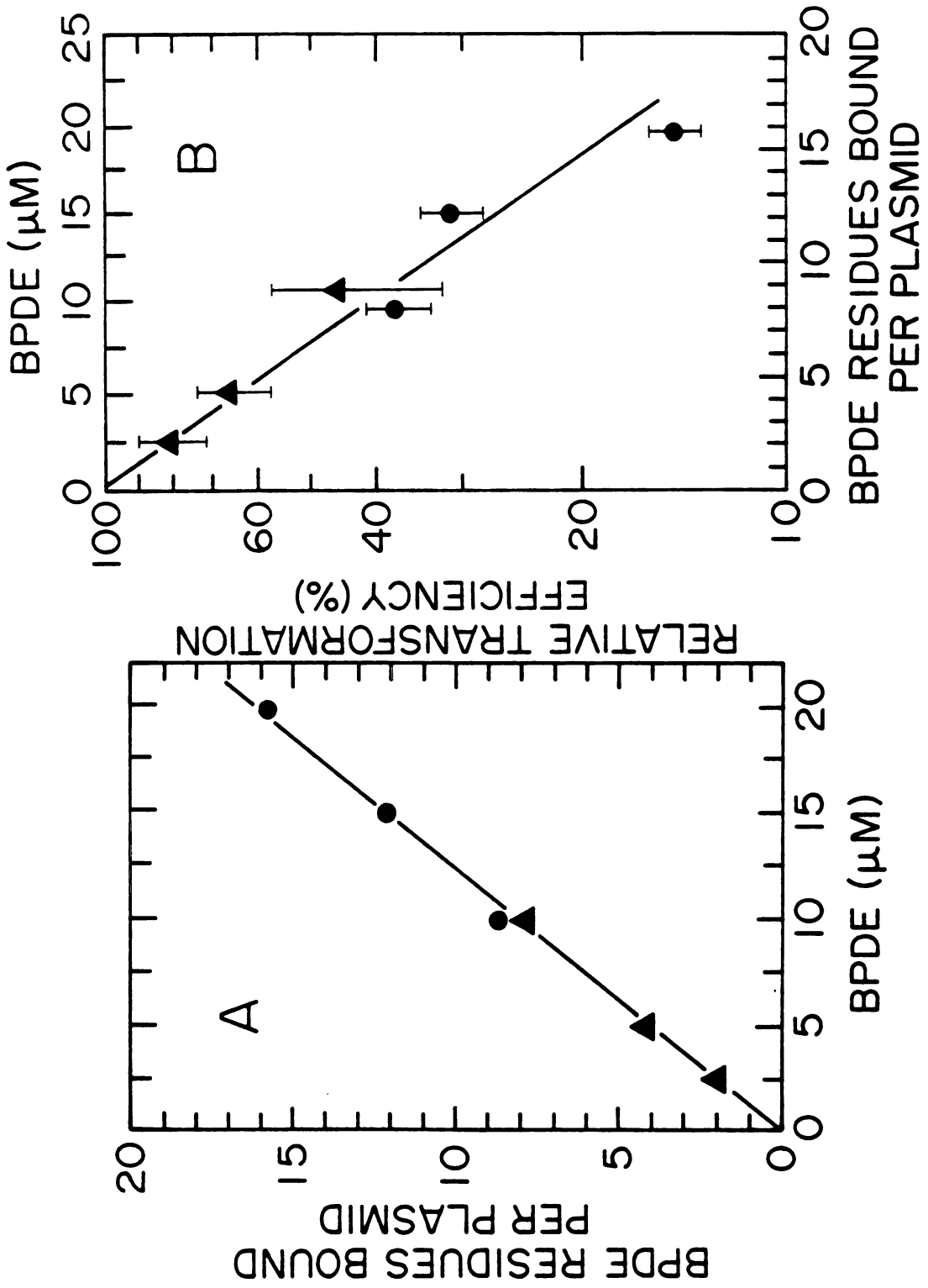
Characterization of Mutants. Plasmid DNA from cells from white or light-blue colonies was amplified, extracted, dissolved in buffer, and analyzed by electrophoresis on 0.8% agarose gels for altered DNA mobility (gross alterations). Plasmids without evidence of gross alterations were amplified further and purified by CsCl centrifugation and analyzed by a secondary bacterial transformation to ensure that the cells' inability to utilize X-Gal actually reflected inactivation of the *supF* gene (see *Results*) If it did, these mutants were sequenced using the dideoxyribonucleotide method of Sanger *et al*. (21) with the following modifications. Plasmids were denatured with alkali to generate single-strand templates (22) and polymerization from a pBR322 *Eco*RI site primer was carried out with the Klenow fragment of DNA polymerase I. Both were purchased from Boehringer Mannheim. ^{35}S-labeled adenosine 5'-[α-thio]triphosphate (New England Nuclear, 034S) and buffer gradient polyacrylamide gels were used for greater resolution (23).

RESULTS

Characterization of BPDE-treated plasmids. pZ189 was treated with BPDE at various concentrations and assayed for the number of residues bound per plasmid and for loss of ability to transform SY204 cells to ampicillin resistance. As shown in Fig. 1, there was a linear relationship between the number of BPDE-induced adducts per plasmid and the BPDE concentration used, and the decrease in transforming activity was directly proportional to the number of BPDE residues bound. Nine residues per pZ189 (1.6 per 10^3 bp) were required to lower the transforming activity to 37% of the control.

Frequency of *supF* Mutants Induced by BPDE Treatment. BPDE-treated and untreated plasmids were transfected into the human cells and allowed to replicate. The progeny plasmids were rescued and introduced into *E. coli* SY204 cells to identify those carrying mutations in the *supF* gene. Fourteen transformation experiments using progeny of untreated plasmid yielded 48 white or light-blue colonies out of 218,750 transformants, an apparent background frequency of 2.2×10^{-4}. However, when plasmid from these colonies was retested by a secondary transformation study in SY204, 17 proved not to have a mutant *supF* gene, indicating that the inability of the bacteria to utilize X-Gal resulted from a separate mutation in the bacteria, not from the lack of an active suppressor tRNA. The frequency of these non-*supF* mutants was 0.8×10^{-4}. Therefore, the background frequency of *supF* mutants among the progeny of plasmids replicating in the human cells was actually 1.4×10^{-4}. Agarose gel and DNA sequence analysis of these mutants showed that 70% of the spontaneous *supF* mutants contained deletions or

Figure 1. (A) Number of BPDE residues bound per plasmid as a function of concentration of BPDE. The symbols (● and ▲) simply indicate that the plasmid was treated with BPDE in two separate experiments. (B) Relative frequency of transformation of bacteria to ampicillin resistance by plasmid pZ189 modified with BPDE compared to untreated plasmid. The error bars indicate the SEM of four or five determinations.


A
BPDE RESIDUES BOUND PER PLASMID
BPDE (μM)
0
5
10
15
20
B
RELATIVE TRANSFORMATION EFFICIENCY (%)
BPDE (μM)
0
5
10
15
20
25
100
60
40
20
0
BPDE RESIDUES BOUND PER PLASMID
0
5
10
15
20

insertions or had altered gel mobility (gross alteration). Therefore, the background frequency of point mutation mutants was only 0.4 x 10^{-4} (9/218,750) (Table 1).

As shown in Table 1 and Fig. 2, the frequency of supF mutants generated during replication of BPDE-treated plasmid in 293 cells increased as a linear function of BPDE residues per pZ189, reaching 38 x 10^{-4} for plasmids containing an average of 16 adducts. Agarose gel and DNA sequence analysis of these mutants showed that none of the plasmids giving high frequencies (i.e., 22.9 x 10^{-4} or 37.8 x 10^{-4}) exhibited deletions and only one contained an insertion (20 bp) (Table 1). Therefore, the frequency of point mutations in the progeny of the pZ189 treated with BPDE at the two highest concentrations was 60 and 95 times higher than background, respectively. Even the supF mutants derived from plasmids exposed to lower doses of BPDE—i.e., those containing 2.0 to 8.7 residues per plasmid, exhibited a much lower proportion of deletion, insertion or altered gel mobility than did those derived from untreated plasmid.

Evidence from two kinds of control experiments indicated that the supF mutants observed were generated in the human cells rather than in the bacteria. First, aliquots of plasmid isolated from human cells and treated with DpnI before being used to transform bacteria to ampicillin resistance gave a high yield of transformants. But when an aliquot of the same Hirt supernatant plasmid preparation was treated with MboI, the number of transformants was drastically reduced—e.g., from 1200 to 1. MboI digests plasmids containing the mammalian cell replication pattern. A second proof comes from experiments in which the indicator bacteria were transformed directly, using the original input

Table 1. Analysis of mutants obtained by transformation of E. coli with progeny of pZ189 generated during replication in 293 cells.

Adducts per plasmid	No. of human cell transfection experiments	supF mutants*/ transformants	Frequency of supF mutants (x 10^4)	Plasmids with altered gel mobility**/ no. examined	Total plasmid supF genes sequenced	Characterization of sequenced mutants			Frequency of supF point mutation♦ x 10^4
						No. with deletions#	No. with insertions"	No. with point mutations+	
0	14	31/218,750	1.4	5/30	25	13	3	9	0.4
2.0	3	40/57,685	6.9	3/36	30	11	0	19	4.0
4.1	3	23/34,030	6.8	6/23	17	2	0	15	4.4
8.7	3	16/25,650	6.2	2/14	11	2	0	9	4.4
7.9	2	18/9,810	18.3	3/11	6	0	0	6	13.3
12.2	2	18/7,850	22.9	0/18	18	0	1	17	21.7
15.6	2	12/3,175	37.8	0/12	11	0	0	11	37.8

*Plasmid from each mutant was assayed by a secondary transformation to ensure that the cell's inability to metabolize X-Gal resulted from inactivation of the supF gene (see text).

**Alteration visible on agarose gel (>150 bp).

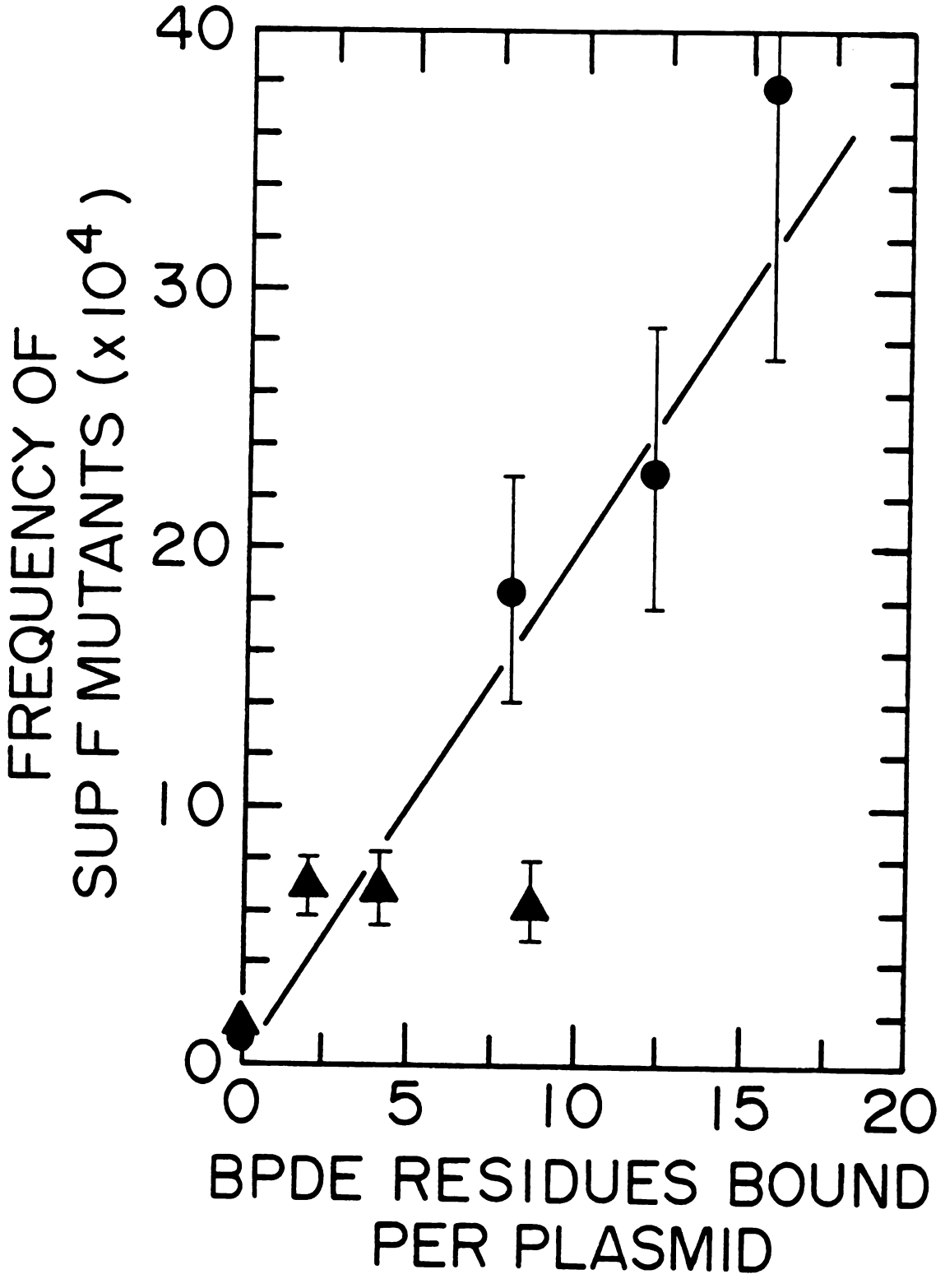
♦Calculated from fraction of point mutants times the observed frequency (column 4). The fraction of point mutants is the number in column 9 divided by that in column 6 plus those mutants showing altered gel mobility (numerator, column 5).

#Deletion of 9 - 150 bp.

"Insertion of 10 - 20 bp.

+Substitution, deletion, or insertion of 1 or 2 bp.

Figure 2. Frequency of supF mutants as a function of the number of BPDE residues per plasmid. Untreated plasmids or plasmids containing BPDE residues were transfected into 293 cells and allowed 48 hr for replication. The progeny plasmids were rescued, treated with DpnI, and transferred into SY204 indicator bacteria by DNA transformation. The frequency of transformants with a non-functioning supF gene were identified by colony color and secondary transformation (see text). The symbols used correspond to those shown in Fig. 1. The error bars refer to SEM of the supF mutant frequencies obtained from a series of individual human cell transfection experiments made with each set of treated plasmid.


FREQUENCY OF
SUP F MUTANTS (x10⁴)
40
30
20
10
0
0
5
10
15
20
BPDE RESIDUES BOUND
PER PLASMID

BPDE-treated plasmid rather than progeny. Untreated plasmid gave a background frequency of 1 white colony per 10^4 total colonies; plasmid containing the highest number of BPDE residues, 15.8, gave only 2 per 10^4. We did not determine what fraction of the latter were true supF mutants.

Characterization of the Mutations Formed in 293 Cells. DNA sequence analysis of the supF gene was carried out on 25 mutant progeny from untreated plasmids and 93 mutant progeny from BPDE treated plasmids (Table 1). Some of the 118 mutants sequenced could have been siblings. However, at least 107 of them, 21/25 from untreated pZ189 and 86/93 from BPDE-treated pZ189, represented unequivocally independent mutants. This is because either their alteration was unique or they had not been derived from the same set of transfected human cells. Therefore, in analyzing the frequency of specific kinds of mutations (Tables 2 and 3) we included only the unequivocally independent mutants. The data showed that 62% (13/21) of the mutants from the control contained deletions or insertions of more than 4 bp, compared to only 17% (15/86) from the BPDE-treated plasmids (Table 2). In the latter, the majority of the deletions were found with mutants produced when the plasmid contained only 2 BPDE-induced adducts (Table 1). Table 2 shows the frequency of specific types of point mutations as well as deletions and insertions of more than 4 bp.

The frequency at which a particular transversion or transition occurred is shown in Table 3. The majority of the changes were single base substitutions. Transversions of a G·C pair to a T·A pair predominated, 6/7 (86%) for the untreated, 45/71 (63%) for the progeny of BPDE-treated plasmid. Transitions occurred much less frequently.

Table 2. Analysis of sequence alterations generated in the *supF* gene by replication BPDE-treated or untreated pZ189 in 293 cells.

	No. of times occurring	
Sequence alterations observed	Untreated	BPDE-treated
Single base substitution	3	51
Two base substitutions		
Tandem	0	3
≤20 bases apart	2	3
>20 bases apart	0	3
Deletions		
Single G·C pair	2	5
Single A·T pair	1	0
Tandem base pairs	0	2
4-20 base pairs	4	3
>20 base pairs	7	11
Insertions		
Single A·T pair	0	4
≤20 base pairs	2	1
Total Sequenced	21	86

Table 3. Kinds of base substitutions generated in the *supF* gene by replication of BPDE-treated or untreated pZ189 in 293 cells.

Base change	No. of mutations observed	
	Untreated	BPDE-treated
Transversions		
G·C → T·A	6	45
G·C → C·G	1	13
A·T → T·A	0	3
A·T → C·G	0	0
Transitions		
G·C → A·T	0	6
A·T → G·C	0	4
Total	7	71

This was the case for mutants obtained with pZ189 containing a low, medium, or high number of BPDE induced adducts. There was no relationship between adduct numbers and the location and types of base substitution mutations.

Mutational Hot Spots for BPDE Induced Mutations. Fig. 3 shows the spectrum of BPDE-induced point mutations. Two strong hot spots were found, position 109 and 123. Both of these sites are located at the middle base pair of GGG triplets of the tRNA gene, and the sites occur opposite each other at the stem of the dihydrouracil loop on the cloverleaf structure of the supF tRNA (Fig. 4). All the base substitutions at position 109 were transversions (10 G·C → T·A, 1 G·C → C·G), 8/10 base substitutions at position 123 were G·C → T·A transversions. As can be seen from Fig. 3, five less prominent hot spots were found at positions 112, 133, 139, 160, and 164. The majority of these also were transversions. Their location on the cloverleaf structure of the supF tRNA are indicated in Fig. 4. Six out of 7 of the BPDE-induced hot spots were located at G·C pairs of the supF gene, and one involved an A·T pair in the center of a G·C-rich region.

The locations of the 10 point mutations obtained from untreated plasmid were distributed over the 85 base pairs of the tRNA gene. Only 1 out of 7 base substitution mutations was located at a hot spot for BPDE-induced substitutions (position 109).

Figure 3. Location of independent point mutations in the *supF* tRNA gene of pZ189. The DNA strand shown is the 5′→ 3′ strand synthesized during the DNA sequencing reaction using the *Eco*RI rightward-sequencing primer. The point mutations observed in the progeny of untreated pZ189 are placed above the tRNA sequence; those from BPDE-treated plasmid are placed below the sequence. A rectangle represents a deleted base; the caret shows the location of an inserted adenine. Every 10th residue and the anticodon triplet are underlined. The tandem deletion of CT, shown at position 133 - 134, could have occurred anywhere between positions 131 and 134.

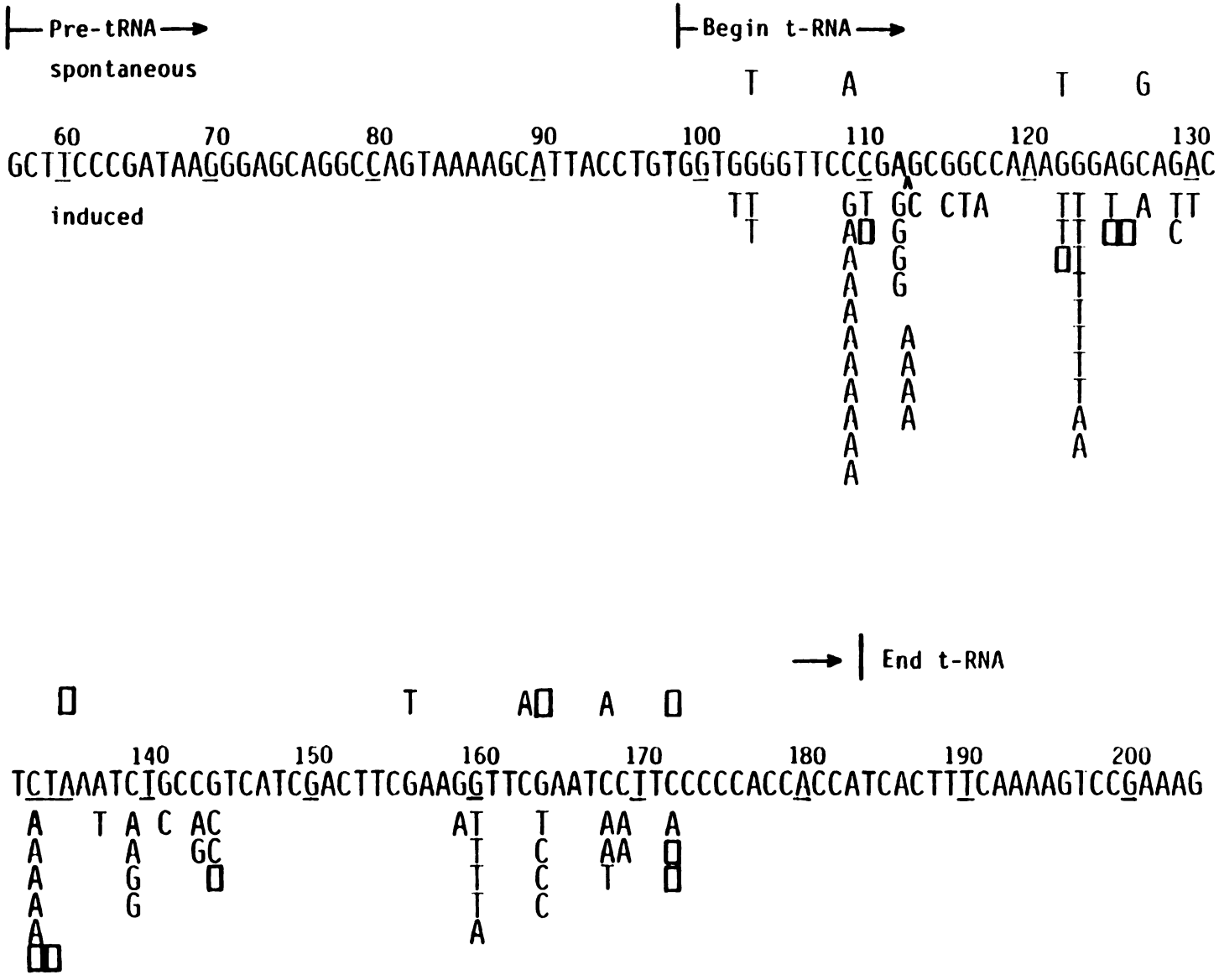

Pre-tRNA
spontaneous
induced
Begin t-RNA
End t-RNA
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
GCTTCCCGATAAGGGAGCAGGCCAGTAAAAGCATTACCTGTGGTGGGGTTCCCGAGCGGCCAAAGGGAGCAGAC
TCTAAATCTGCCGTCATCGACTTCGAAGGTTCGAATCCTTCCCCCACCACCATCACTTTCAAAAGTCCGAAAG

Figure 4. Location of the BPDE-induced hot spots on the cloverleaf structure of the supF tRNA. The circles indicate the two very strong hot spots, each of which exhibited 10 or 11 base substitution mutations out of a total of 64. The squares indicate the 5 less-hot spots, each of which exhibited 4 or 5 base substitutions, and the hexagon indicates a site at which 3 base changes occurred.

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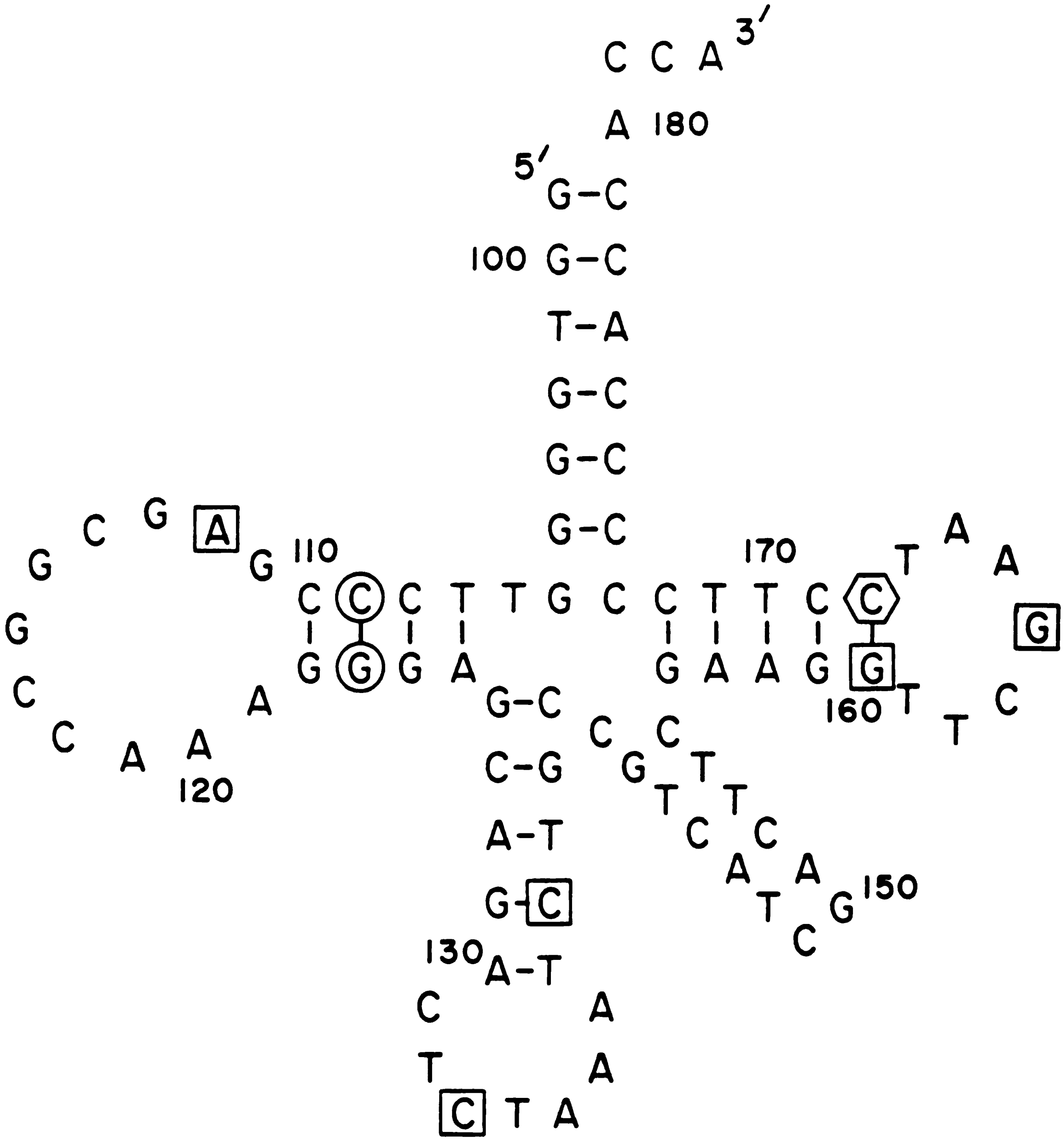

DISCUSSION

The data in Tables 1-3 indicate that BPDE causes a decrease in the frequency of gross rearrangements, deletions, and insertions in plasmids rescued from the human cells and a corresponding increase in the frequency of point mutations. It is known that transfection of shuttle vectors into mammalian cells results in a high frequency of spontaneous deletions and insertions (1,2,13,24-28). When rodent or monkey cell lines are used, the frequencies can exceed 1% (13). Since high backgrounds can obscure the induction of mutations by a specific mutagen, it is important to reduce this effect. Calos and her associates (13) have shown that use of the 293 cell line results in a significantly lower background of lacI mutants compared to monkey cell lines. We also found that the frequency of spontaneous supF mutants formed when pZ189 replicated in COS7 monkey cells was higher than in 293 cells (data not shown).

Since the observed gross rearrangement mutations are hypothesized to involve nucleolytic degradation of the plasmid, ligation and, perhaps, homologous recombination (13,27), Seidman and coworkers (4) engineered the pZ189 plasmid so that the supF gene is strategically located between two genes essential for replicating in the bacterial host. This means that plasmids containing gross rearrangements—e.g., deletions that extend from supF into either of the neighboring genes, are not recovered in the indicator bacteria. The combined use of the supF gene in pZ189 and 293 cells for our study resulted in the low background frequency we observed, 1.4×10^{-4} with 0.4×10^{-4} consisting of point mutations. The fact that the progeny of BPDE-treated plasmid

rescued from human cells exhibited a much lower frequency of gross rearrangements, deletions, and insertions than did the control suggests that the presence of BPDE adducts interferes with the cellular processes that cause gross alterations.

The data in Table 3 indicate that 90% of the BPDE-induced base substitutions involved G·C base pairs. This result correlates with the reported high percentage of binding of BPDE to guanine (29). The predominant site for adduct formation by BPDE in double-stranded DNA is the N^2 position of guanine (11), but binding to the N-7 position of guanine can occur (30,31). The N^2 guanine adduct is stable, but the N-7 guanine adduct can result in depurination, leaving an alkali labile site. Loeb and Preston (32) have suggested that apurinic or apyrimidinic sites are the lesions responsible for transversions. If so, the principal potentially mutagenic BPDE lesion should be the unstable N-7 guanine adduct. Information bearing on the question has recently been provided by Lobanenkov et al. (14) who examined the sequence specificity of single-strand breakage induced by treating double-stranded DNA with BPDE. They showed that scission at dG residues is sequence specific and that cleavage and/or the generation of alkali sensitive apurinic sites occurs preferentially at triplets in which the middle dG has a pyrimidine on the 5′ side. In their study, all of the TGA, TGT, TGG, CGT, and CGG sites exhibited a cleavage. Cleavage at the middle dG when a purine was at the 5′ side was much rarer, with 5′-AGT-3′ being cleaved, but not 5′-GGA-3′ or 5′-GGC-3′, and cleavage at the dG of 5′-AGC-3′ required a C to the 5′ of A. Cleavage of the middle dG of a GGG triplet was very rare. They concluded that cleavage at sequence-specific sites results from the

nonrandom formation of unstable BPDE adducts.

Although they did not study the mutagenic effects of BPDE, these investigators hypothesized that the marked effect of neighboring bases on the frequency of G-specific DNA cleavage may be responsible for BPDE-induced mutational hot spots. Analysis of our data on the specific location of the base substitution mutations in the sequence of the supF gene (Fig. 3) indicates that this is not the case. Neither of our two major hot spots was a G with a pyrimidine located 5′ to it. In fact, of all the base substitutions we observed that involved a G·C pair, only 20% (13/64) occurred at sites predicted by the study of Lobanenkov et al. (14) to be the location of an unstable guanine adduct.

In view of the above arguments, our data support the hypothesis that the N^2 guanine adduct is the principal potentially mutagenic BPDE-induced lesion. The reason for the seven hot spots shown in Fig. 4 cannot be that only those areas of the gene respond to base changes, since the data obtained in the present study, combined with that of previously published studies (6,7,10,12), show that a change in any one of at least 63 of the 85 bases in the tRNA structure will result in a phenotypic change. It should be noted that, except for position 123, the mutagenic hot spots found in the supF tRNA gene after BPDE treatment differ from those found with UV treatment (6). This is not surprising, since UV damage involves pyrimidines rather than purines.

Our finding that BPDE treatment of pZ189 and its subsequent replication in human cells results in point mutations, predominantly base substitutions, agrees with the results of a comparative genetic marker study by Aust et al. (33) and the Southern blot analysis of King

and Brookes (34). Our data showing that the majority of the transversions were G·C → T·A support the findings of Eisenstadt *et al.* (35) in bacteria. They used genetic crosses to analyze a large number of nonsense mutations in the *lacI* gene for the kinds of base substitutions induced by BPDE in *E. coli*. Our studies also agree with the more limited data reported earlier (36-38) on the kinds of mutations formed when BPDE-treated plasmids replicate in bacteria or in monkey cells (28).

As shown in Table 3, 79% (48/61) of the transversions obtained with progeny of BPDE-treated plasmids involved a change to a T·A. The majority (45/48) were G·C → T·A. This preference supports the hypothesis that the DNA polymerase involved preferentially inserts adenine across from a noninstructional base (39), but transversions to C·G were also found (13/61). In their study with *E. coli*, Eisenstadt *et al.* (35) who found similar results, invoked the purine pairing model proposed by Topal and Fresco (40) to explain how such transversions could occur. Such purine-purine mispairing may also be occurring in mammalian cells.

ACKNOWLEDGEMENT

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CHAPTER V

Kinds and Spectrum of Mutations Induced by 1-Nitrosopyrene Adducts during Plasmid Replication in Human Cells

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SUMMARY

1-Nitropyrene (1-NP) has been shown in bacterial assays to be the principal mutagenic agent in diesel emission particulate. It has also been shown to be mutagenic in human fibroblasts and carcinogenic in animals. To investigate the kinds of mutations induced by this carcinogen, and compare them with those induced by a structurally related carcinogen, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetra hydrobenzo[a]pyrene (BPDE) (Yang et al. Proc. Natl. Acad. Sci. USA, 84:3787,1987), we treated a shuttle vector with tritiated 1-nitrosopyrene (1-NOP), a carcinogenic, mutagenic intermediate metabolite of 1-NP which forms the same DNA adduct as the parent compound, and introduced it into human cells for DNA replication to take place. The treated plasmid, pZ189, carrying a bacterial suppressor tRNA target gene supF, was allowed 48 hr to replicate in the human cells. Progeny plasmids were then rescued, purified, and introduced into bacteria carrying an amber mutation in the -galactosidase gene in order to detect those carrying mutations in the supF gene. The frequency of mutants increased in direct proportion to the number of DNA-1-NOP adducts formed per plasmid. At the highest level of adduct formation tested, the frequency of supF mutants was 26 times higher than the background frequency of 1.4×10^{-4}. DNA sequencing of 60 unequivocally independent mutants derived from 1-NOP-treated plasmid indicated that 80% contained a single base substitution, 5% had two base substitutions, and 4% had small insertions or deletions (one or two base pairs), and only 11% showed a deletion or insertion of four or more base pairs. Sequence data from

25 supF mutants derived from untreated plasmid showed 64% contained deletions of 4 or more base pairs. The majority (83%) of the base substitutions in mutants from 1-NOP-treated plasmid were transversions, with 73% of these being G·C → T·A. This is very similar to what we found previously in this system using BPDE, but each carcinogen produced its own spectrum of mutations in the supF gene. Of the five hot spots for base substitution mutations produced in the supF gene with 1-NOP, two were the same as seen with BPDE-treated plasmid. However, the three other hot spots were cold spots for BPDE-treated plasmid. Conversely, the four other hot spots seen with BPDE-treated plasmid were cold spots for 1-NOP-treated plasmid. Comparison of the two carcinogens for the frequency of supF mutants induced per DNA adduct showed that 1-NOP adducts were 3.5 times less effective than BPDE adducts.

INTRODUCTION

Many carcinogens have been found to induce tumors only at specific sites in the body of the treated animal. For example, i.p. injection of benzo[a]pyrene, a widely distributed polycyclic aromatic hydrocarbon formed by incomplete combustion, forms tumors predominantly in the lung (24,39), whereas the structurally-related nitro aromatic compound 1-nitropyrene (1-NP) primarily forms liver (39) or mammary tumors (14). One explanation for such specificity is that the carcinogen is metabolized into a reactive form only in specific target organs. Another is that the carcinogen is capable of inducing only certain kinds of mutations and that a specific change in DNA is required in a particular organ to bring about the necessary alteration leading ultimately to tumorigenesis. The latter hypothesis is supported by recent studies on the specific mutational changes needed to activate specific oncogenes (7,37,43). Therefore, we and others (3,5,8,9,16-18,21,22,30,32,40,41) have begun to study the specific kinds of mutations induced by carcinogens.

Attempts to deduce the nature of the molecular mechanisms by which carcinogens induce mutations in mammalian cells have previously been hampered by the inability to isolate and analyze newly mutated genes at the sequence level. But the development of shuttle vectors, i.e., plasmids carrying a defined target gene and capable of replicating in mammalian cells and also in bacteria, provide a solution to this problem (8,9,21,32,33). We are using the shuttle vector pZ189, containing the *supF* gene which codes for a tyrosine suppressor tRNA, to investigate at the DNA sequence level the kinds of

mutations induced when DNA containing covalently bound carcinogen residues replicates in human cells. The advantage of using the supF gene as the target for mutation studies at the sequence level is its small size and the fact that it is highly responsive to base changes. Examination of data from a number of studies, including those in refs. 3,5,6,17,18,30,40,41 indicate that a change in any one of at least 63 of the 85 bases which make up the tRNA structure will result in a mutant phenotype. Use of the human cell line 293 as the eukaryotic host for replicating the plasmid offers the advantage of a background mutant frequency of 1.4×10^{-4} (41) which is low enough to allow one to observe an increase in mutant frequency induced by carcinogen treatment of the plasmid.

Using this system, we recently determined the specific kinds of mutations induced by the major reactive metabolite of benzo[a]pyrene, (±)-7β,8α-dihydroxy-9α, 10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) (41), which has been shown to form lung tumors when i.p. injected into newborn mice (24). In the present study we investigated the mutagenic specificity of 1-nitrosopyrene (1-NOP), a partially reduced metabolite of 1-NP, which forms the same DNA adducts as the parent compound (1,29), induces mutations in bacteria (12) and mammalian cells (13,28,29), and forms liver tumors in animals (39). Both 1-NOP and BPDE form covalently bound adducts in DNA principally with guanine (12,19,26,38). 1-NOP binds only at the C8 position (1,12,13,29), whereas BPDE forms its principal guanine adduct at the N^2 position (38). Since these guanine adducts are located in very different positions in the DNA helix and only one, the N^2 position, is involved in the base pairing part of the molecule, we were particularly

interested in comparing the specific kinds of mutations induced by these two agents, their location in the target gene, as well as their biologic effectiveness, i.e., their ability to induce mutations when the treated plasmid replicates in human cells and to interfere with bacterial transformation. The results indicate that the adducts formed by the two carcinogens are equivalent in decreasing bacterial transformation and the kinds of mutations induced by 1-NOP adducts are similar to those induced by BPDE. However, the frequency of mutants induced per DNA adduct is 3.5 times lower for 1-NOP than for BPDE, and each compound exhibits a specific spectrum of base changes in the target gene.

MATERIALS AND METHODS

Cells and Plasmid. The human embryonic kidney cell line, 293, was grown in modified Eagle's minimal essential medium containing 10% fetal calf serum (Grand Island Biological Co., Grand Island, NY) as described previously (41). The ampicillin sensitive indicator bacterial host was, E. coli SY204, which carries an amber mutation in the β-galactosidase gene (32). The 5.5 kbp shuttle vector, pZ189, contains the supF tyrosine suppressor tRNA gene flanked by two genes essential for recovery in E. coli, i.e., the ampicillin gene and the bacterial origin of replication (33). It also carries the origin of replication and large T-antigen gene from simian virus-40.

Formation of 1-NOP adducts on the plasmid. Plasmid DNA, prepared using an alkaline lysis procedure (25) and purified by ethidium bromide-CsCl density centrifugation, was resuspended in 190 µl of helium-purged sodium citrate (10 mM) buffer, pH 5.0, at a concentration of 300 µg/ml. A 5 µl aliquot of a freshly prepared solution of ascorbic acid (20 µM in H_2O) was added to provide needed reduction of the 1-NOP (12), followed by 5 µl of a stock solution of tritiated 1-NOP (specific activity 217 mCi/mmole, purity 99%) dissolved in dimethylsulfoxide at a concentration of 0.05 to 0.08 µM. The radiolabeled 1-NOP was supplied by Dr. F. A. Beland of the National Center for Toxicological Research, Jefferson, AK. The samples were immediately mixed and incubated at 37°C for 2 hr. Unbound 1-NOP was removed by phenol-chloroform extraction and three successive ethanol precipitations. The moles of 1-NOP residues bound per mole of plasmid was calculated from the A_{260} absorption profile of the DNA and the

specific activity.

Transfection and rescue of replicated plasmid. The human cell line 293 was plated into a series of 150 mm-diameter dishes at 1 x 10^6 cells per dish. After 24 hr the cells in each dish were transfected with 6 µg of plasmid using a modification of the $CaPO_4$ coprecipitation technique as described (41). After 48 hr, the cells from each dish were harvested separately and progeny plasmid was extracted and separated from cellular DNA as described (15,41). The DNA was purified with buffered phenol, treated with RNase A (50 µg/ml) at 37°C for 1 hr, followed by proteinase K (100 µg/ml) at 50°C for 2 hr, then extracted with phenol-chloroform, precipitated with ethanol, and further purified by drop dialysis (34). In order to distinguish between independent mutants with identical mutations and putative siblings derived from a single event, the progeny plasmid obtained from each dish of cells was maintained and assayed separately. Before progeny plasmid was used to transform indicator bacteria, it was treated with 20 units of DpnI, a restriction enzyme which digests any input DNA that still has the bacterial methylation pattern generated when the plasmid was first prepared in bacteria. As a control, an aliquot was also treated with MboI, which digests plasmid generated during replication in human cells, and tested for any residual transforming ability. The results showed that >99.9% of the purified plasmid was derived from material that had replicated in the human cells.

Bacterial transformation and mutant identification. The progeny plasmid DNA was assayed for mutant supF genes by transforming E. coli SY204 to ampicillin resistance and selecting the transformants on plates containing ampicillin (50 mg/ml), X-Gal, and an inducer of the

β-galactosidase gene, as described (41). Transformants containing plasmids lacking a functioning supF gene, which is needed to suppress the amber mutation in the β-galactosidase gene of the bacteria, could be identified because they form white or light blue colonies rather than dark blue colonies on X-Gal plates. Each white or light blue putative mutant colony was restreaked on fresh plates to confirm the phenotype.

Characterization of mutants. Plasmid DNA from bacteria in these white or light blue colonies was amplified, extracted, dissolved in buffer, and analyzed by electrophoresis on 0.8% agarose gels for altered DNA mobility (gross alteration). Plasmids without evidence of gross alterations were amplified further and purified by CsCl centrifugation and analyzed by a secondary bacterial transformation to ensure that the observed inability of the bacteria to utilize X-Gal was the result of inactivation of the supF gene, rather than a mutation in the β-galactosidase gene of E. coli (41). Putative supF mutants were sequenced using the dideoxyribonucleotide method (31) modified as follows. Plasmids were denatured with alkali to generate single-stranded templates (42) and polymerization from a pBR322 EcoRI site primer was carried out using the Klenow fragment of DNA polymerase I. The primer and polymerase were purchased from New England Biolabs (Beverly, MA). ^{35}S-αdATP (New England Nuclear, NEN-034S, Boston, MA) and buffer gradient denatured polyacrylamide gels (2) were used for greater resolution of the sequencing gel.

Determination of sites of carcinogen-induced adducts. The positions of 1-NOP- or BPDE-adducts in the supF gene of carcinogen-treated plasmid were determined by the in vitro DNA polymerase-stop

assay of Moore and Strauss (27). Briefly, double-stranded plasmid containing 1-NOP- or BPDE adducts (10 to 70 adducts per molecule of plasmid) 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, so that the average number of adducts per strand of the supF gene was 0.2 to 1.5. The polymerization reaction were then carried out as for the sequencing reaction except that the dideoxynucleotides were omitted. 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 autoradiography were determined by a laser densitometer (LKB 2222-010, Ultroscan XL) and were corrected for position in the gene by taking into account the number of ^{35}S-labeled adenine bases that would be present in each length of newly synthesized DNA.

RESULTS

Characterization of 1-NOP-treated plasmid. Vector pZ189 was treated with various concentrations of tritium labeled 1-NOP in the presence of ascorbic acid as the reducing agent. The number of residues bound per plasmid was determined and their ability to interfere with transformation of bacterial cells to ampicillin resistance was determined using the transformation method of Hanahan (11). As shown in Figure 1, the number of 1-NOP-induced adducts per molecule of plasmid increased linearly with the concentration of 1-NOP used. Figure 2 shows that the transforming activity of modified plasmid decreased in direct proportion to the number of 1-NOP residues bound. Approximately seven 1-NOP residues bound were required to lower the transforming activity of the treated plasmid to 37% of the untreated control plasmid.

Frequency of mutants induced by 1-NOP adducts. Plasmid containing various levels of 1-NOP adducts and untreated plasmid were introduced into human cells by transfection and allowed to replicate for 48 hr. The progeny plasmid were harvested and assayed for mutations in the supF gene by introducing them into E. coli SY204 indicator bacteria. As shown in Figure 3, there was a linear increase in the frequency of supF mutants as a function of the number of 1-NOP-induced adducts per plasmid. At the highest level of 1-NOP adduct formation tested, i.e., 63 adducts per pZ189, the frequency of supF mutants was 35.8×10^{-4}, which is 26 times higher than that the background frequency of 1.4×10^{-4}.

Agarose gel and DNA sequence analysis of mutants obtained with 1-

Figure 1. Number of 1-NOP adducts bound per plasmid as a function of concentration of 1-NOP in the presence of ascorbic acid (0.5 uM). The symbols (● and ▲) indicate that the plasmid was treated with 1-NOP in two separate experiments.

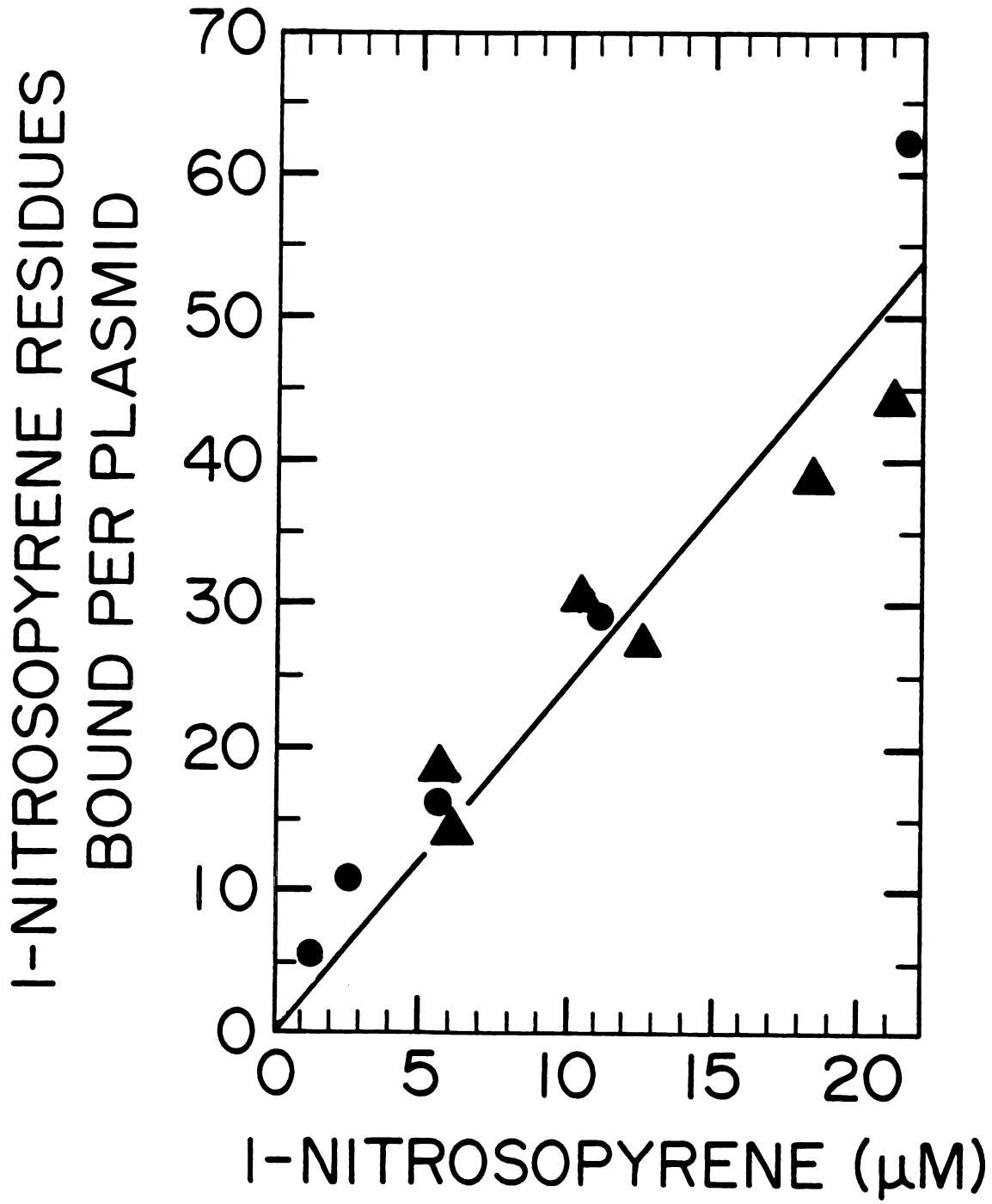

1-NITROSOPYRENE RESIDUES BOUND PER PLASMID
70
60
50
40
30
20
10
0
0
5
10
15
20
1-NITROSOPYRENE (μM)

Figure 2. Relative frequency of transformation of bacteria to ampicillin resistance by plasmid pZ189 containing 1-NOP adducts compared to untreated plasmid. The error bars indicate the SEM of four determinations.

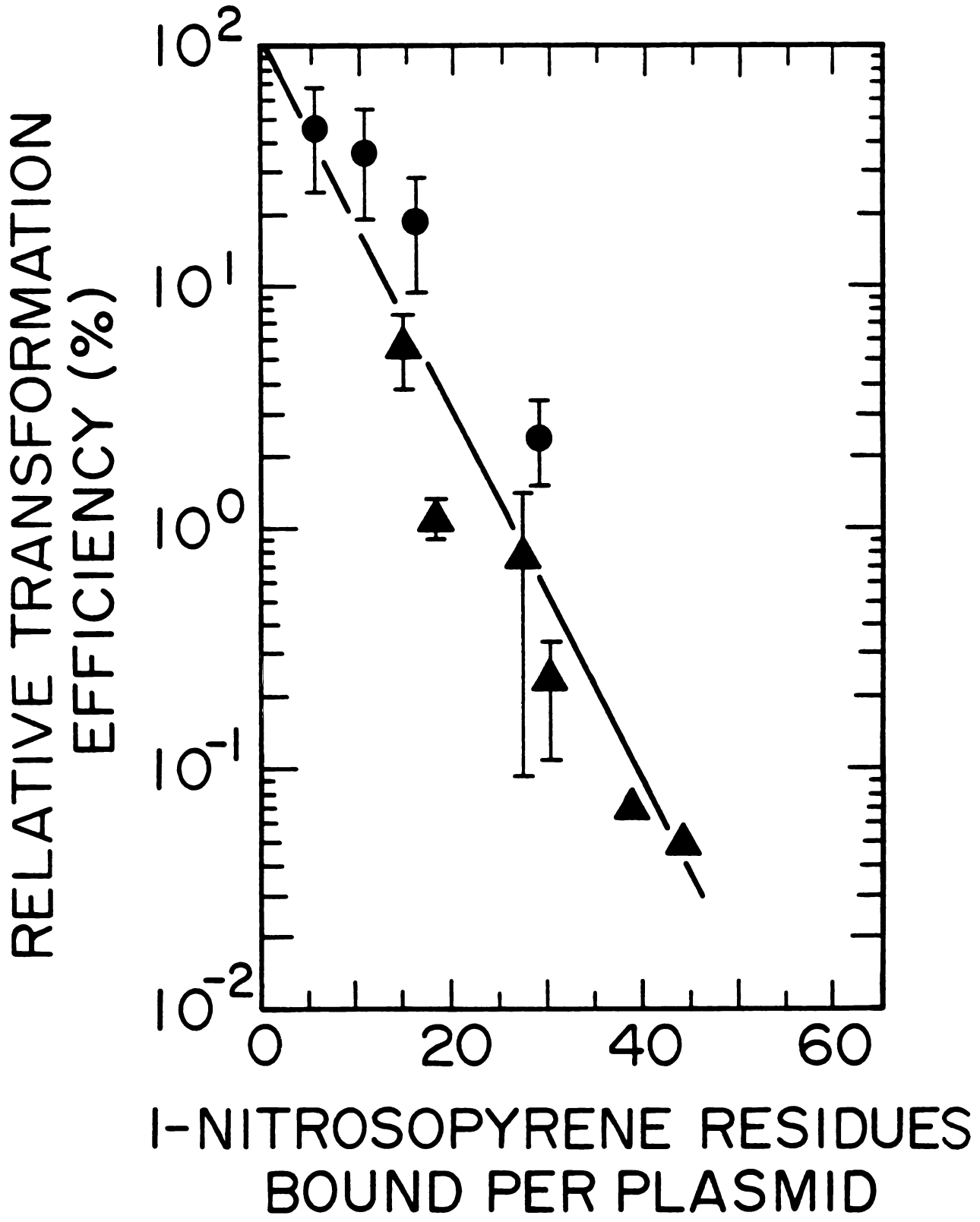
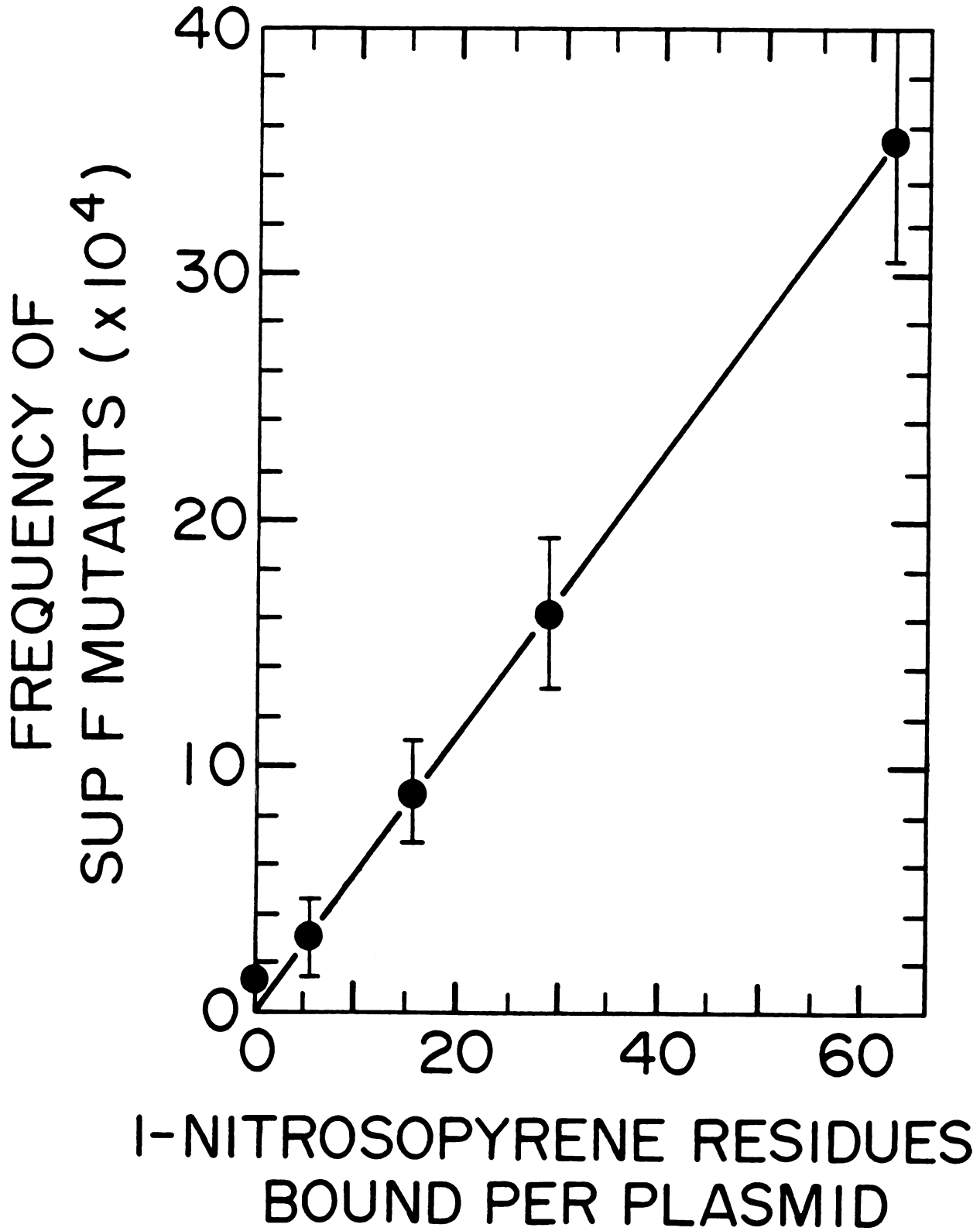

RELATIVE TRANSFORMATION EFFICIENCY (%)
10^2
10^1
10^0
10^-1
10^-2
0
20
40
60
1-NITROSOPYRENE RESIDUES BOUND PER PLASMID

Figure 3. Frequency of supF mutants as a function of the number of 1-NOP adducts per plasmid. Untreated plasmids or plasmids containing 1-NOP adducts were transfected into 293 cells and allowed 48 hr for replication. The progeny plasmids were rescued, treated with DpnI, and introduced into indicator bacteria by DNA transformation. The frequency of transformants with a nonfunctioning gene was identified by colony color and secondary transformation (see text). The error bars refer to SEM of the supF mutant frequencies obtained from a series of individual human cell transfection experiments made with each set of treated plasmid.


40
30
20
10
0
FREQUENCY OF
SUP F MUTANTS (x 10⁴)
0
20
40
60
1-NITROSOPYRENE RESIDUES
BOUND PER PLASMID

NOP-treated plasmid revealed that the majority of them contained point mutations. The data are summarized in Table 1. Included in the table are data from 14 experiments in which human cells were transfected with untreated plasmid to determine the background frequency of supF mutants and to generate control mutants for analysis. Eight of these control transfection experiments with untreated plasmid accompanied studies with BPDE-treated plasmid, six accompanied studies with 1-NOP-treated plasmid. The analysis of all 31 of the mutants obtained was reported in our earlier paper showing the mutagenic effect of BPDE (41), but the data have been included here in Table 1 for comparative purposes.

The mutants derived from plasmids containing the two highest levels of adducts contined mainly point mutations. Only 4.5% (3/67) exhibited altered gel mobility, and only 10% of those mutants that were sequenced (5/50) proved to contain deletions or insertions of four or more base pairs. In contrast, 70% of the control mutants (21/30) contained such mutations. Calculation of the fraction of mutants containing point mutations (Table 1, column 10) showed that the frequencies derived from plasmid carrying the two highest levels of 1-NOP adducts were respectively 35 and 75 times higher than the control.

Nature and location of the specific mutations induced by 1-NOP-treated plasmid in 293 cells. We examined the nature of the point mutations induced in plasmids carrying 1-NOP adducts, as well as the location of the point mutations in the target gene. As shown in Table 2, sequence analysis of 60 unequivocally independent mutants derived from 1-NOP-treated plasmid indicated that 88.3% contained point mutations, i.e., 80% (48/60) contained a single base substitution, 5% (3/60) contained two base substitutions and 3.3% (2/60) had deletion

Table 1. Analysis of mutants obtained by transformation of *E. coli* with progeny of 1-NOP-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 (x 10^4)	Plasmids with altered gel mobility[b]/ no. examined	Total plasmid *supF* genes sequenced	Characterization of sequenced mutants: No. with deletions[d]	No. with insertions[e]	No. with point mutations[f]	Frequency of *supF* point mutation[c] x 10^4
0	14	31/218,750	1.4	5/30	25	13	3	9	0.4
5.7	2	4/12,985	3.1	0/4	4	1	0	3	2.3
16.2	4	18/20,200	8.9	3/17	10	1	0	9	6.2
29.1	4	27/16,705	16.2	1/26	20	2	0	18	13.9
62.5	4	42/11,720	35.8	2/41	30	1	2	27	30.2

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

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

[c]Calculated from fraction of point mutants times the observed frequency (column 4). The fraction of point mutants is the number in column 9 divided by that in column 6 plus those mutants showing altered gel mobility (numerator, column 5).

[d]Deletion of 9 - 150 bp.

[e]Insertion of 10 - 20 bp.

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

Table 2. Analysis of sequence alterations generated in the *supF* gene by replication 1-NOP-treated or untreated pZ189 in 293 cells.

	No. of times occurring	
Sequence alterations	Untreated	1-NOP-treated
Single base substitution	3	48
Two base substitutions		
Tandem	0	2
≤20 bases apart	2	0
>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 base pairs	4	3
>20 base pairs	7	2
Insertions		
Single A·T pair	0	1
≤20 base pairs	2	2
Total sequenced	21	60

or insertion of one base pair. These data contrast with the control mutants where only 38% showed such point mutations. Table 3 shows the specific kinds of base substitutions induced in the supF gene by replication of 1-NOP-treated plasmid in 293 cells. The majority (45/54) of the changes were transversions, with 33 out of 45 being G·C → T·A. The majority (87%) of base changes (47/54) involved G·C pairs.

The specific location of 1-NOP-induced point mutations (spectrum) is shown in Figure 4. Three strong hot spots were found at positions 109, 123 and 127. All of the base substitutions at these three hot spots were G·C pair transversions (16 G·C → T·A, 4 G·C → C·G). Two less prominent hot spots were seen at positions 156 and 159 with transitions occurring twice as frequently as transversions (6 G·C→ A·T, 3 G·C → T·A). All five hot spots were located at the stem of the cloverleaf structure of the supF tRNA (Figure 5). All seven of the A·T pair base substitutions were located in the loops of the tRNA cloverleaf structure.

Sites of 1-NOP or BPDE-adducts. To determine whether there was a correlation between the positions and frequencies of the carcinogen-induced mutations and the sites and frequencies of carcinogen-DNA adducts in the supF gene, we carried out the DNA synthesis-stop assay of Moore and Strauss (27), in which bulky adducts interfere with DNA replication. The template DNA used in the assay contained 10 to 70 1-NOP or BPDE adducts per plasmid, which represents 0.2 to 1.5 adducts per strand of the supF gene, if binding to guanine is essentially random. This protocol for estimating the percentage of carcinogen adducts formed at particular sites on the gene assumes first, that the density of the bands in a sequencing gel, adjusted for extent of

Table 3. Kinds of base substitutions generated by replication of 1-NOP-treated or untreated pZ189 in 293 cells.

Base change	No. of mutations observed	
	Untreated	1-NOP-treated
Transversions		
G·C → T·A	6	33
G·C → C·G	1	8
A·T → T·A	0	3
A·T → C·G	0	1
Transitions		
G·C → A·T	0	6
A·T → G·C	0	3
Total	7	54

Figure 4. Location of independent point mutations in the supF tRNA gene of pZ189. The DNA strand shown is the 85 nucleotides making up the tRNA structure. The point mutations observed in the progeny of 1-NOP treated plasmid are placed below the sequence. The rectangle represents a deleted guanine; the caret shows the location of an inserted thymidine. Every tenth residue and the anticodon triplet are underlined.

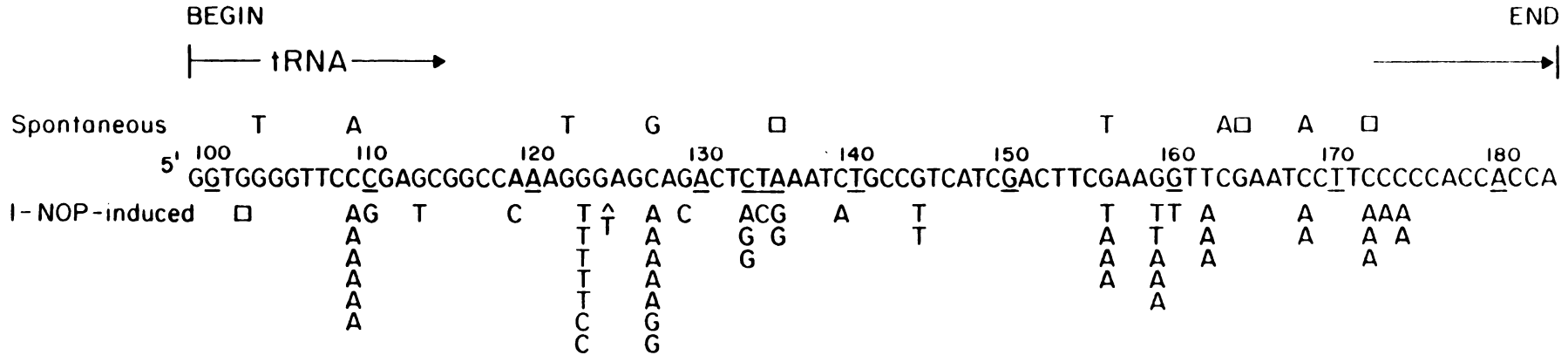
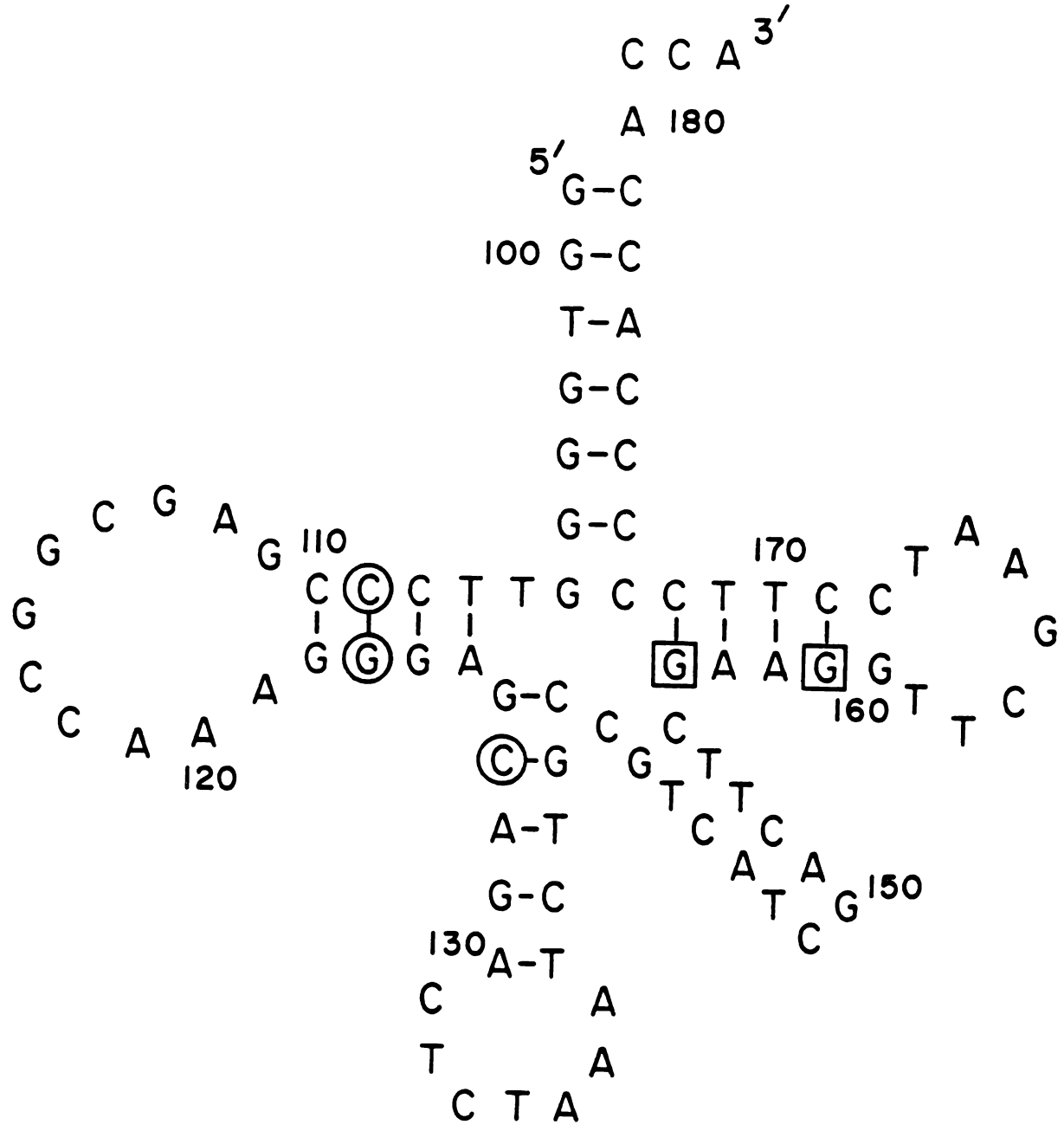

BEGIN
END
tRNA
Spontaneous
1-NOP-induced
5'
100
110
120
130
140
150
160
170
180
GGTGGGGTTCCCGAGCGGCCAAAGGGAGCAGACTCTAAATCTGCCGTCATCGACTTCGAAGGTTCGAATCCTTCCCCCACCACCA

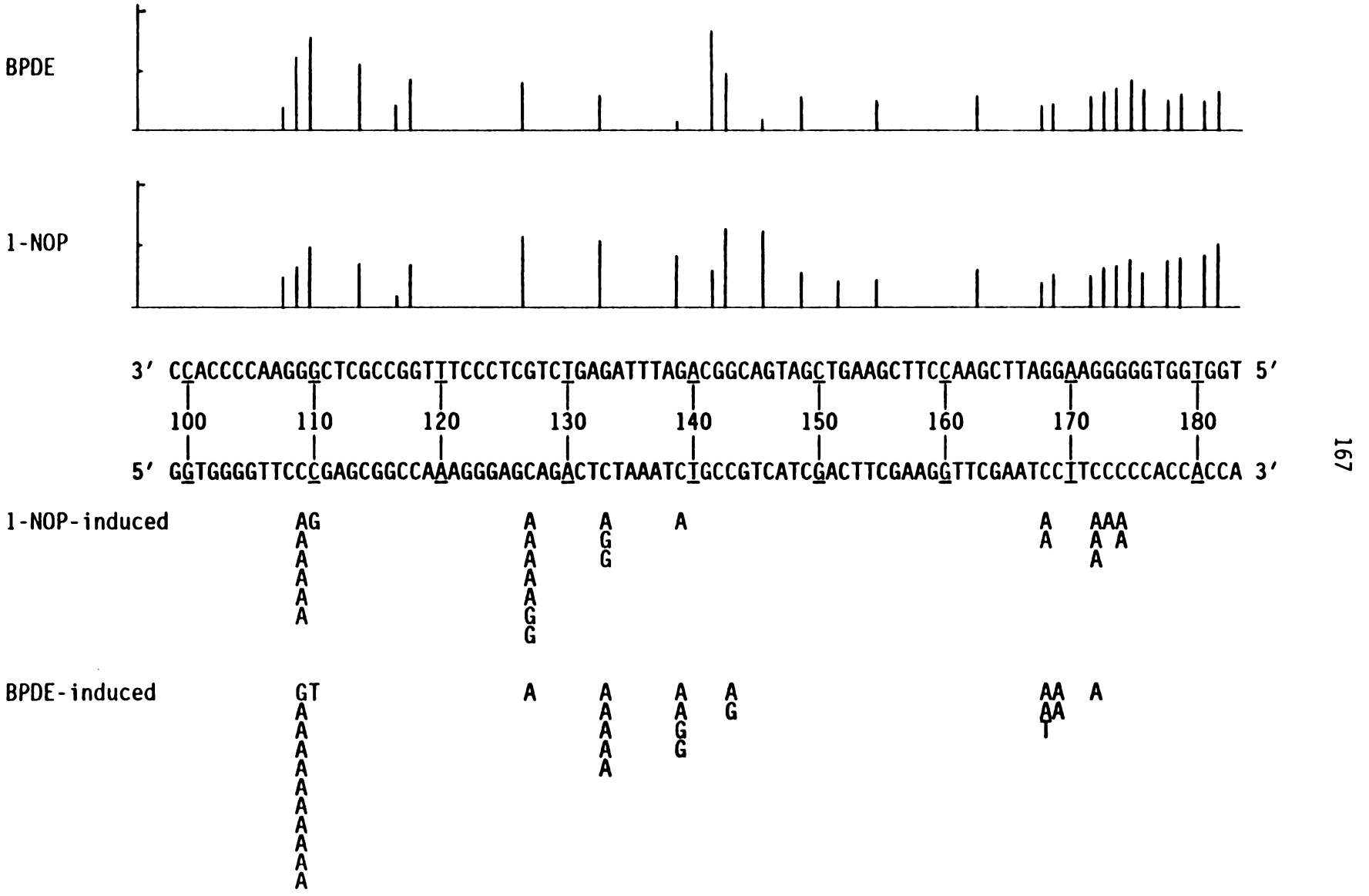
Figure 5. Location of the 1-NOP-induced hot spots on the cloverleaf structure of the supF tRNA. The circles indicate the three strong hot spots. The squares indicate the 2 less prominent hot spots.


C C A 3′
A 180
5′
G–C
100 G–C
T–A
G–C
G–C
G–C
C G A
G G 110
G
C A
C A A
120
C C C T T G C C T T C C
G G G A
G A A G G
170 T A
160 T T C
G
G–C
C–G
A–T
G–C
130 A–T
C A
T A
C T A
C C
G T
T T
C C
A A
T G 150
C

^{35}S-αdATP incorporated, is proportional to the number of DNA molecules of a particular length, and second, that the length reflects the chance of adduct-induced premature termination of the polymerization.

The gel pattern of the DNA bands obtained in this assay for DNA containing 1-NOP or BPDE residues corresponded to positions one nucleotide before virtually every cytosine residue in the DNA sequencing standard lane, indicating that DNA synthesis was terminated one band away from each guanine in the template. No evidence of any interference with polymerization was obtained with untreated template and bands corresponding to positions one nucleotide away from any base other than guanine were not seen. The pattern of bands did not vary significantly with the numbers of 1-NOP- or BPDE-adducts per molecule of plasmid. Presumably, these bands were generated by a stop of the Klenow fragment of DNA polymerase I at the sites of bulky adducts. The relative intensities of the bands in the 85 nucleotides making up the tRNA of the 3′ to 5′ template using the pBR322 EcoRI site primer are shown in Figure 6. The frequency of DNA adducts, as estimated with this assay, ranged from 0% (position 131) to 6.6% (position 143) for 1-NOP-adducts and 0% (position 131 and 152) to 12.9% (position 110) for BPDE-adducts. As shown in Figure 6, the 1-NOP-guanine adducts were most frequently located at position 143, a position at which no mutation was observed. The frequency of 1-NOP-guanine adducts located at positions 109, 114, 118, 142, 149, 163, and 169 was approximately the same (~3%), but only at 109 did mutation occur and, in fact, position 109 represents a very strong mutational hot spot for 1-NOP. This apparent lack of correlation between the frequency of adduct formation and mutation frequency was also found with BPDE.

Figure 6. Relative frequency of 1-NOP-guanine or BPDE-guanine adducts in the supF gene of pZ189 and location of 1-NOP- or BPDE-induced base substitutions within the tRNA coding sequence of pZ189. Klenow fragment of DNA polymerase I was used with EcoRI rightward sequencing primer to determine polymerase-stop sites on the 3′ to 5′ strand of 1-NOP- or BPDE-treated supF gene (panel shown above the supF gene sequence). The relative intensities of the bands on the autoradiography were determined by densitometer and were corrected for position in the gene by taking into account the number of ^{35}S-labeled adenine bases that would be present in each length of newly synthesized DNA. The base substitutions shown below the supF tRNA gene were G·C base changes on the 5′ to 3′ supF gene using the EcoRI rightward site primer.


BPDE
1-NOP
3′ CCACCCCAAGGGCTCGCCGGTTTCCCTCGTCTGAGATTTAGACGGCAGTAGCTGAAGCTTCCAAGCTTAGGAAGGGGGTGGTGGT 5′
100
110
120
130
140
150
160
170
180
5′ GGTGGGGTTCCCGAGCGGCCAAAGGGAGCAGACTCTAAATCTGCCGTCATCGACTTCGAAGGTTCGAATCCTTCCCCCACCACCA 3′
1-NOP-induced
BPDE-induced

DISCUSSION

The data in Fig. 1 indicate that the number of 1-NOP adducts per molecule of plasmid increased linearly with the concentration of 1-NOP used in the presence of ascorbic acid. The amount bound per applied concentration of carcinogen was three times higher than what we found previously with BPDE (41), but unlike the direct-acting BPDE, 1-NOP requires reducing agents such as ascorbic acid for activation (12). Once the 1-NOP adducts formed, they were very similar to BPDE adducts in their ability to interfere with the processes involved in bacterial transformation, i.e., approximately seven 1-NOP adducts per plasmid (Figure 2) and nine BPDE adducts per plasmid (41) were sufficient to lower the transforming activity of the treated plasmid to 37% of that of untreated plasmid.

The data in Table 3, indicating that 1-NOP adducts caused base substitutions mainly at G·C base pairs (87%) just like BPDE does (41), strongly suggust that the mutagenesis was targeted to sites where adducts occur. Targeted mutagenesis is also indicated for 1-NOP-induced base substitutions involving A·T base pairs. Our results (Table 3) show that 13% of the base changes involved A·T base pairs, and Kinouchi and Ohnishi (19) recently demonstrated with bacterial nitroreductases that a minor adduct formed by 1-NP involved deoxyadenosine. We consider the base substitutions induced by these two carcinogens to be targeted to adducts rather than to apurinic sites, as suggested by Loeb and Preston (23). The argument for this conclusion for BPDE was given in our earlier paper (41). In the case of 1-NOP, apurinic sites are highly unlikely since this carcinogen

does not form unstable adducts.

The majority of the 1-NOP or BPDE-induced base changes we observed were G·C → T·A transversions. It may be that, as suggested by Strauss et al. (35), the DNA polymerase in the human 293 cell line preferentially inserts an adenine nucleotide opposite a nonstructional base containing a bulky adduct ("A rule"). However, G·C → C·G transversions were also found (15% of the base substitutions). Another explanation for the predominance of G·C → T·A transversions, and the one which we favor, is that with some frequency, purine·purine base pairing occurs when the guanine carries a 1-NOP residue (or a BPDE residue). Eisenstadt et al. (10) invoked the model of Topal and Fresco (36), in which such mispairing occurs by having the guanine carrying a BPDE residue at the N^2 position be oriented in the syn position and pair with an incoming adenine in the rare tautomeric imino form. However, Kennard and her colleagues (4) recently reported that stable $G_{anti}{\cdot}A_{syn}$ base pairs are formed in a synthetic deoxydodecamer and are accommodated in the DNA double helix with little or no disruption of the local or global conformation. Their model does not require the formation of the very rare enol or imino tautomeric forms. 1-NOP binds predominantly to the C8 position of guanine which is not involved in base pairing. The presence of this bulky residue may direct guanine in the normal anti configuration to pair with an incoming adenine nucleotide triphosphate with adenine adopting the syn configuration. This would result in a stable base pair.

Using the polymerase-stop assay (Figure 6), we did not find a high correlation between the frequency of 1-NOP or BPDE adduct sites and the frequency of base substitution mutations at these sites. Although the

"A rule" or/and the purine·purine mispairing mechanism may explain G·C → T·A transversions, they cannot easily explain the location of the mutational hot spots we observed with either carcinogen. This lack of correlation is not caused by the inability of changes in these sites to cause detectable phenotypic changes in the tRNA gene since base substitutions at most of these positions have been detected previously. Fuchs and his coworkers proposed the "mutation-prone sequences" model to explain the lack of correlation between the sites of acetylaminofluorine-induced frameshift mutations in a bacterial plasmid and the sites at which T4 DNA polymerase exonuclease III activity was blocked by acetylaminofluorine residues. Similarly, Brash et al. (3) proposed the "pass/fail" model to explain the lack of correlation of mutation frequency with UV-induced photoproduct frequency at different sites in the supF gene. Such "mutation-prone sequences"- or "pass site"-determined mutational hot spots, rather than mere DNA lesion-determined hot spots, also fit our findings, but do not explain why some sites are very hot for mutation occurrence.

Although both 1-NOP and BPDE induced point mutations, with single base substitutions predominating and with G·C → T·A transversions being the most common mutation, the spectrum of mutations in the supF tRNA gene induced by 1-NOP differed from that induced by BPDE. The spectrum of the two carcinogens exhibited two hot spots in common (positions 109 and 123). However, three 1-NOP-induced hot spots were cold spots for BPDE (positions 127, 156, and 159), and four BPDE-induced hot spots were cold spots for 1-NOP (positions 112, 139, 160, and 164). These differences are not likely to be the result of differential binding of the two carcinogens at those positions, at

least not as determined by the DNA polymerase-stop assay. For example, adducts were formed at position 139 more frequently by 1-NOP than by BPDE, but this site was a mutational hot spot for BPDE not for 1-NOP. These differences may, as suggested by Brash et al. (3), reflect the fact that structural features differ between hot spots and non-hot spots in DNA sequences and that the ability of carcinogen-modified nucleotides to alter DNA structure at specific sites differs for 1-NOP and BPDE.

This explanation is consistent with our finding that when the mutagenicity of these two structurally-related carcinogens is compared on the basis of equal numbers of adducts per plasmid, 1-NOP is 3.5 times less effective than BPDE in inducing mutations during the replication of the modified plasmid in the human host cells. This difference in mutagenic effectiveness of these two carcinogens may reflect the intrinsic difference in the nature of the adducts formed. However, it may also reflect a difference in the rate of excision 1-NOP- or BPDE-adducts from the plasmid by the host 293 cells. Unpublished data from this laboratory indicate that diploid human fibroblasts can excise 1-NOP-induced adducts faster than BPDE-induced adducts (V. M. Maher, J. D. Patton and J. J. McCormick, manuscript in preparation). Therefore, the ability of 293 cells to remove 1-NOP- or BPDE-adducts from DNA is currently under investigation.

It may be important to point out that, unlike Seidman, Kraemer and their collaborators who studied UV-induced supF mutations in pZ189 replicating in SV40-transformed human cells (3,5), or Hauser et al. who studied this in a monkey cell line (18), we did not observe multiple mutations occurring in a single mutant plasmid. The reason

for this significant difference in results between UV induced lesions and adducts formed by bulky carcinogens is also currently under investigation in our laboratory.

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