Molecular mechanisms of mutations induced by environmental pollutants, 3-nitrobenzanthrone, crotonaldehyde and acrolein

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PREFACE

The study presented in this thesis has been carried out under the directions of Professor Saburo Matsui at Research Center for Environmental Quality Control, Kyoto University and Professor Hiraku Takebe* at Department of Radiation Genetics, Faculty of Medicine, Kyoto University during 1996 - 1998. Throughout the course of this research, the author has had the continuous good fortune to be in contact with people who provided useful suggestions, encouragement and aids of many kinds. The author would like to express his gratitude to all of them for their many contributions to the success of this work.

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CONTENTS

GENERAL INTRODUCTION

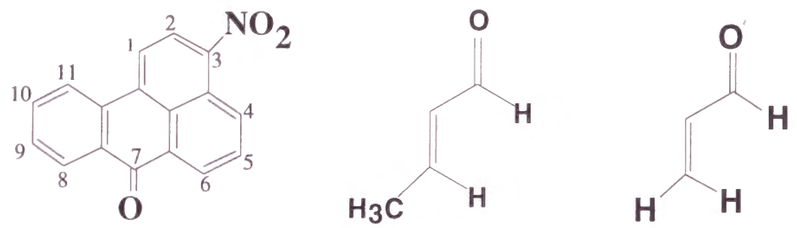
Natural and synthetically-produced chemicals represent a major paradox in our modern world. Millions of different chemical substances are used daily around the world, and have proven to be very beneficial to mankind in many years. Unfortunately, there are also undesirable consequences associated with the use of these chemicals, often with hazardous or toxic effects on humans. In recent years, genetic toxicity of some of these chemicals has been revealed by the methods of molecular biology.

It has been believed that deoxyribonucleic acid (DNA) is the informationally active chemical component of essentially all genetic materials and it was assumed that this macromolecule must be extraordinarily stable maintaining high degree of fidelity required for the master blueprint. It has been revealed, however, that the primary structure of DNA is in fact quite dynamic and subject to constant change. Many of these changes arise as a consequence of errors during replication, recombination and repair of DNA. Base alterations may also arise from the inherent instability of specific chemical bonds that constitute the normal nucleotides under physiological temperature and pH conditions. DNA of living cells often reacts with various chemical compounds and physical agents (*e.g.*, X-rays), many of which are present in the environment. Some of these chemicals are products of the metabolism or decomposition of other living forms with which many organisms exist in intimate proximity. Others, particularly in recent decades, are man-made and contribute to genetic insult faced by individuals living in highly industrialized communities (1).

An agent that leads to an increase in the frequency of mutation, 'mutagen', either acts on DNA directly to change its template properties or in some way subvert replication so that a wrong base is inserted. DNA reacting chemicals act directly on DNA to change the bases into chemically distinct structures. These new bases often base pair in a different way from the original bases, in which case the effect of mutagen is to alter the genetic code directly (2). In recent years, an increasing public awareness of environmental mutagens has led to an intense interest in studying the mechanisms by which genotoxic chemicals interact with and damage DNA. One of the goals in this thesis is to illustrate the interaction of environmental chemicals with DNA.

Mutagen may produce mutagen-specific base alterations as fingerprints in DNA, with respect to the nature of the changes, locations of the changes, and frequencies of the alterations in the gene. Therefore, an analysis of the spectrum of mutation may provide an answer through the empirical approach to the fundamental question: What causes genetic changes in cells ? It is now well established that changes in the specific DNA base sequences in the cancer-related genes lead to cancer. Some of the chemical carcinogens have been shown to yield the specific fingerprints of DNA damage. Therefore, to identify the spectra of mutation induced by carcinogenic chemicals which are present in the environment should contribute in evaluating their carcinogenic or mutagenic risks.

In this thesis the author focused on three environmental mutagens, 3-nitrobenzanthrone (NBA, 3-nitro-*7H*-benz[*d,e*]anthracen-7-one), crotonaldehyde and acrolein (Figure A). These compounds are air pollutants in urban area and



3-nitrobenzanthrone crotonaldehyde acrolein

Figure A. Structures of 3-nitrobenzanthrone, crotonaldehyde and acrolein

virtually human population is exposed to them regularly.

NBA is a new class of powerful bacterial mutagen and suspected human carcinogen recently found in diesel exhaust and airborne particulates (3). Its mutagenicity by Ames *Salmonella* assay is very high (208,000 revertants / nmol in *Salmonella typhimurium* TA98 and 6,290,000 revertants / nmol in YG1024) and compares with that of 1,8-dinitropyrene (1,8-DNP) which is the direct mutagen of strongest activity so far reported (3). This new mutagen is also shown to induce micronuclei in mouse peripheral blood reticulocytes, suggesting its potential genotoxicity to mammals (3). However, any other study on biological effect of NBA has not been available.

Crotonaldehyde and acrolein are the simplest member of the class of α,β-unsaturated carbonyl compounds. These are products of combustion; automobile emissions, forest fires and urban fires, and cigarette smoke all contain significant quantities. These chemicals are mutagenic to bacteria *Salmonella typhimurium* (4,5). Although simple in structure, these compounds exhibit a rich chemistry of DNA modification. The structures of the DNA adducts produced by the chemicals has been well elucidated (6). Deoxyguanosine residues appear to be most reactive to the compounds and the chemicals produce hydroxy-1,N^2-propanodeoxyguanosine (7). Despite the well understanding of their DNA damages, mutational specificities of them are still unknown.

For better understanding of mutagenicity of these chemicals, the author intends to donor the fundamental data of their genotoxicity. The aim of this research is to elucidate the molecular mechanisms of mutations induced by NBA, crotonaldehyde

and acrolein. The specific objectives are as follows:

1. Identify the chemical structures of DNA damage (adduct) induced by NBA
2. Reveal the mutational specificities of NBA, crotonaldehyde and acrolein

Survey of this thesis

Chapter 1 concerns with the structure of DNA adduct formed by NBA. By use of a chemically-synthesized reactive derivative of NBA, the structure of NBA-DNA adduct was identified as *N*-acetyl-3-amino-2-(2'-deoxyguanosin-8-yl)-benzanthrone.

Chapter 2 deals with the investigation of DNA adducts formed in human cells treated with NBA. The result of Chapter 1 is based on *in vitro* study. In order to construct a bridge linked *in vitro* to *in vivo*, the author examined the formation of the adduct in the human cells. ^{32}P-postlabeling analysis revealed that the adduct exists in the cells. This result indicates that the result of Chapter 1 is able to be applicable to phenomena *in vivo*.

Chapter 3 describes the mutational specificity of NBA in human cells using the chemically-synthesized reactive derivative of NBA. The derivative induced mainly G:C to T:A base substitutions, and its specificity was similar to those by other nitroaromatic carcinogens.

Chapters 4 and 5 concern with mutational spectra of crotonaldehyde and acrolein, respectively. Both compounds induced frequently G:C to T:A base substitutions in human cells.

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CHAPTER 1

An unusual DNA adduct derived from the powerfully mutagenic environmental contaminant 3-nitrobenzanthrone

Abstract

The covalent binding of an *N*-oxy metabolite of the powerfully mutagenic 3-nitrobenzanthrone (NBA) to 2'-deoxyguanosine (dG) and calf thymus DNA has been investigated *in vitro*. The major adduct obtained from the reaction of *N*-acetoxy-*N*-acetyl derivative (*N*-Aco-*N*-Ac-ABA) of 3-aminobenzanthrone (ABA) and dG was identified as *N*-acetyl-3-amino-2-(2'-deoxyguanosin-8-yl)benzanthrone (dG-*N*-Ac-ABA) by ^{1}H NMR and mass spectroscopies. The adduct also formed in the reaction of *N*-Aco-*N*-Ac-ABA with calf thymus DNA. The coupling with dG moiety occurred exclusively at C-2 of benzanthrone (BA), suggesting a significant contribution of a resonance-stabilized arenium ion intermediate derived from BA to the production of this new type of adduct.

Introduction

Nitrated polycyclic aromatic hydrocarbons and their derivatives (nitro-PAH) are widespread environmental pollutants (1-4). Some members of this class of compounds are known to be mutagenic in bacterial and mammalian cells and tumorigenic in rodents (3-5). Recently, 3-nitrobenzanthrone (3-nitro-*7H*-benz[*d, e*]anthracen-7-one; NBA) has been found to be a new class of powerful mutagen present in air borne particulates and/or diesel exhaust particles (6). The direct-acting mutagenicity of this compound in *Salmonella typhimurium* TA98 is comparable to that of 1,8-dinitropyrene, which is the strongest direct-acting mutagen so far reported in the literature. NBA has also been demonstrated to induce micronuclei in mouse peripheral blood reticulocytes after intraperitoneal administration, suggesting its potential as a genotoxin in the mammals (6).

The covalent binding of nitro-PAH metabolites to cellular DNA has been well recognized to play an important role for the initiation of mutagenesis and carcinogenesis (2,5,7). Several mutagenic nitro-PAH are shown both *in vivo* and *in vitro* to be reductively activated to form the DNA adducts predominantly at C-8 or N^2-position of 2'-deoxyguanosine (dG) and to a lesser extent at C-8 and N^6-positions of 2'-deoxyadenosine (dA) (7,8). Alike other known nitro-PAH mutagens, NBA would be subjected to the metabolic activation in order to form a DNA adduct and exert the genotoxic effect (7-9). In a nitroreductase-deficient *Salmonella* strain TA98NR, the direct-acting mutagenic activity of NBA was found to be lower than in TA98, while in strain YG1024 which overproduces acetyltransferase, the mutagenic activity was 30 times as high as in TA98 (6, 10). These results strongly suggest

that NBA, alike other nitro-PAH, undergoes initial metabolic reduction of the nitro group to form an *N*-arylhydroxylamine, which is then esterified to produce a reactive *N*-acetoxy or *N*-sulfonyloxy ester (7,11-14). These activated esters readily undergo the heterolytic N-O cleavage to form a highly reactive nitrenium ion, which combines with a DNA molecule to yield the corresponding DNA adduct (8,9).

The objectives of this Chapter are to elucidate the mechanism of the mutagenesis induced by NBA and also to obtain the NBA-DNA adduct for using as a marker for exposure of cells to NBA. In order to accomplish these objectives, *N*-acetoxy-*N*-acetyl-3-aminobenzanthrone (*N*-Aco-*N*-Ac-ABA) was synthesized and the formation and structure of the addition products from this compound and dG or DNA were investigated.

Materials and methods

Warning: *3-Nitrobenzanthrone, 3-aminobenzanthrone, and their derivatives are all potential carcinogens and therefore should be handled with care.*

General

Melting points were determined on a Yanagimoto hot stage apparatus and are uncorrected. Thin layer chromatography (TLC) was performed by using Merck precoated silica gel sheets 60F-254. Silica gel (Wakogel C-200) was used for column chromatography. IR spectra were recorded on a SHIMADZU FT-IR DR 8000/8100 infrared spectrophotometer and only prominent peaks in 2000-700 cm^{-1} region were recorded. ^{1}H-NMR spectra were obtained in DMSO-d_6 with a Varian Gemini-200 (200 MHz) spectrometer. For the ^{1}H and ^{13}C NMR characterization of the adducts, JEOL-JNM-A500 (500MHz for ^{1}H NMR) and JEOL-JNM-LA300 (75.45MHz for ^{13}C NMR) spectrometers were used. Electron impact mass spectra (EI-MS) were recorded on a SHIMADZU GC-MS QP-2000A spectrometer at 70 eV and chemical ionization mass spectra (CI-MS) on a SHIMADZU GC-MS QP-5000 with DI-50 using isobutane as a reacting gas. Electrospray mass spectra (ES-MS) were determined on a Thermo Quest TSQ-7000 system equipped with a Michrom BioResources UMA model 600 HPLC system. Fast atom bombardment mass spectra (FAB-MS) were recorded with a JEOL MS-MP 7000.

Chromatography

High performance liquid chromatograph (HPLC) analysis and purification of the

adducts were performed using a Waters 600E HPLC system equipped with a Waters 991J photodiode array detector and a reversed-phase column of Waters μbondosphere C_{18}(3.9 x 15 mm). Preparative low pressure liquid chromatograph (LPLC) for the purification of crude adducts was performed with a Yamazen Model 540 equipped with a Model SSC-3000AII UV detector and a FR 50N fraction collector, using a Yamazen ODS-S-40 B semi-preparative column (30 mm i.d. x 250 mm).

Chemicals and Reagents

All solvents were of analytical grade and used after distillation. Reagent grade benzanthrone was purchased from Tokyo Kasei Kogyo Co. Ltd. and used after recrystallization from ethanol (purity >99.9%). 2'-Deoxyguanosine (dG) was purchased from Wako Chemicals Co. Ltd. Calf thymus DNA (Type I), deoxyribonuclease I (DNase I, Type IV) and phosphodiesterase I (Type VII) were obtained from Sigma Chemical Co. Ltd. Alkaline phosphatase was obtained from Toyobo Biochemicals Co. Ltd.

3-Nitrobenzanthrone (NBA)

3-Nitrobenzanthrone was prepared by the nitration of benzanthrone; benzanthrone (2.5 g) was dissolved in nitrobenzene (30 ml) and treated with concentrated HNO_3 (4.0 ml) with stirring at 40 °C for 30 min. Crude NBA separated out as a yellow solid from the cooled reaction mixture and recrystallized successively from glacial acetic acid and dichloromethane.

N-Acetyl-N-hydroxy-3-aminobenzanthrone (N-Ac-N-OH-ABA)

This compound was obtained by the reported method (15). To a suspension of NBA (275 mg) in dimethylformamide (DMF; 20 ml) cooled to 0 °C, 5% palladium /carbon (50 mg) and hydrazine monohydrate (0.2 ml) was added with stirring. The color of the reaction mixture immediately changed from greenish yellow to red, and NBA disappeared gradually as the reaction proceeded. The progress of the reaction was monitored by TLC (hexane - ethyl acetate, 5:1). When the reduction was complete, triethylamine (0.3 ml) followed by acetyl chloride (1.5 ml) was added below 10 °C. After stirring at room temperature for 1 h, the reaction mixture was filtered, diluted with water, and neutralized with aq. sodium carbonate. The solution was extracted with dichloromethane and the resulting organic phase was reextracted with 0.1 M NaOH (20 x 3 ml). The combined aqueous extracts were acidified with concentrated HCl to give the expected product as a yellow solid, pure enough for subsequent purposes. ^{1}H NMR (DMSO-d_6) δ 2.3 (s, 3H), 7.6-7.9 (m, 4H), 8.3-8.4 (m, 2H), 8.6-8.7 (m, 2H), 8.8 (d, J = 8.0, 1H), 10.9 (s, 1H); IR (KBr) λmax 2839, 1637, 1576, 1327 cm^{-1}; FAB-MS m/z 304.0 (M+1); HR-EI-MS $C_{19}H_{13}NO_3$, calcd 303.0895, found 303.0883.

N-Acetoxy-N-acetyl-3-aminobenzanthrone (N-Aco-N-Ac-ABA)

N-Ac-*N*-OH-ABA (200 mg) were dissolved in 5 mL of 2% NaOH. After acetic anhydride (1.2 equiv) was added to the solution, the precipitate was filtered and washed with water. *N*-Aco-*N*-Ac-ABA was obtained as a yellow solid. This solid was used without further purification for the reactions with dG and DNA. For the

mass and NMR inspections, the compound was further purified by column chromatography on silica gel using dichloromethane/ethanol as the eluent. Although most part of *N*-Aco-*N*-Ac-ABA decomposed during elution, a small amount of pure compound could be isolated as a yellow powder. Mp 196 °C (decomp); ^{1}H NMR (DMSO-d_6) δ 1.6 (s, 3H), 2.2 (s, 3H), 7.5 (t, J = 7.2, 1H), 7.7-7.9 (m, 3H), 8.2 (d, J = 8.0, 1H), 8.4-8.6 (m, 3H), 8.7 (d, J = 7.2 1H); UV λmax 276, 284, 310, 396 nm; EI-MS m/z (%) 345 (M+, 4.5), 285 (50.5); HR-EI-MS $C_{21}H_{15}NO_4$, calcd 345.1001, found 345.1010.

N-Acetyl-3-amino-2-(2'-deoxyguanosin-8-yl)benzanthrone (dG-N-Ac-ABA)

N-Aco-*N*-Ac-ABA (280mg) and dG (300mg) were dissolved in acetonitrile /water (1:1, 40 ml) buffered at pH 5 (sodium citrate) or pH 7 (potassium phosphate) and stirred overnight at 60 °C under argon. The reaction mixture was filtered and the filtrate was diluted with acetonitrile to bring about precipitation of the buffer salt and unchanged dG. The precipitate was removed by filtration and the filtrate was evaporated in vacuo to dryness. The residue was washed with a minimum amount of dichloromethane, dissolved in DMF, and subjected to LPLC using a column of ODS (MeOH-H_2O; step gradient, 10 ~ 60%). The fractions containing the adduct were evaporated to dryness and the residue obtained was again subjected to LPLC (MeOH-H_2O containing 0.1% triethylamine; step gradient, 10 ~ 60%, 10mL/min). After evaporation of the solvent in vacuo followed by freeze-drying, dG-*N*-Ac-ABA was obtained as a yellow solid. Further purification for analytical purpose was made with a HPLC system using 10 % methanol in water as the eluent with a linear

gradient to 100% methanol in 120 min at a flow rate of 0.8 ml/min. The fraction eluting at 67.37 min was collected and freeze-dried.

Reaction of N-acetoxy-N-acetyl-3-aminobenzanthrone (N-Aco-N-Ac-ABA) with calf thymus DNA

Calf thymus DNA (Type I, 1.5 mg) was incubated at 50 °C for 15 h in the presence of *N*-Aco-*N*-Ac-ABA (3 mg) in a solution of 10 mM sodium citrate buffer (1.2 ml, pH 5) containing 50% acetonitrile. The reaction mixture was then extracted with ethyl acetate (5 x 0.4 ml). After the addition of one tenth volume of 3 M sodium acetate (pH 5.2), the modified DNA was precipitated by diluting with ice-cold ethanol. The recovered DNA (0.75 mg) was redissolved in a mixture of 0.4 ml of 10 mM Tris-HCl (pH 8) and 1 mM EDTA and enzymatically digested to a mixture of nucleosides; initial incubation with deoxyribonuclease I (0.1 mg/mg of DNA) at 37 °C for 3 h in the presence of 5 mM $MgCl_2$ was followed by the second incubation with alkaline phosphatase (1.5 u/mg of DNA) and phosphodiesterase I (0.12 u/mg of DNA) at 37 °C for 24 h. The hydrolysate was extracted with water-saturated butanol and the extract was washed with butanol-saturated water. The butanol phase was taken up and evaporated to dryness under reduced pressure to leave a residue, which was redissolved in DMSO for HPLC analysis.

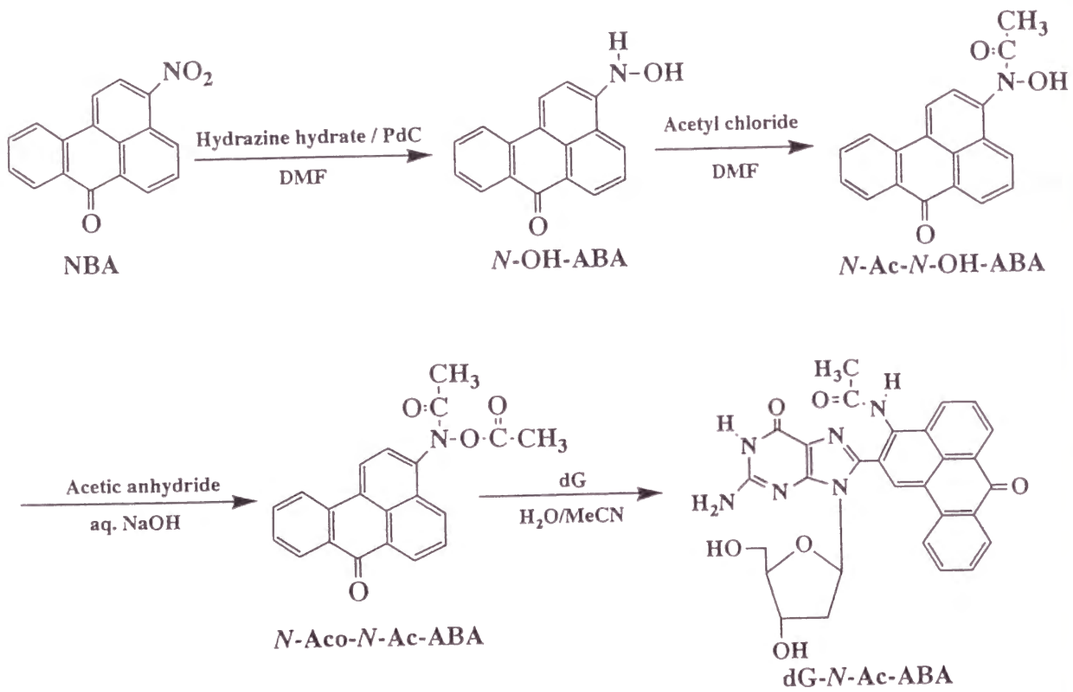
Results

Synthesis of N-Acetoxy Derivative of 3-Aminobenzanthrone (ABA)

For the *in vitro* preparation of nitro-PAH-DNA adducts, several successful approaches have previously been reported. They include the reactions of (i) dG with *N*-acetoxy-*N*-trifluoroacetyl or *N*-acetoxy derivatives of nitro-PAH metabolites (16-20), (ii) dG with *N*-acetyl-*N*-acyloxyamines (21-25), and (iii) DNA with hydroxylamine derivatives in acidic media (26-29). The author choses the *N*-acetoxy-*N*-acetyl route, since *N*-acetoxy-*N*-acetyl and several related derivatives were successfully employed for the synthesis of dG adducts of several aromatic amines (Scheme 1-1) (21-25). Moreover, this compound is relatively stable and easy to handle for the present purpose.

The starting material *N*-Ac-*N*-OH-ABA was efficiently prepared by the Pd/C-catalyzed reduction of NBA with hydrazine hydrate in DMF, followed by selective *N*-acetylation of the resulting hydroxylamine according to the Weatra's method (15); the reduction of NBA to 3-(hydroxyamino)benzanthrone (*N*-OH-ABA) was complete within 30 min and subsequent treatment of the reaction mixture with triethylamine/acetyl chloride gave *N*-Ac-*N*-OH-ABA in 60% yield. When the reduction product mixture was diluted with water, *N*-OH-ABA was obtained as a red precipitate. Attempted reduction of NBA to *N*-OH-ABA in dry THF failed probably due to poor solubility of NBA.

Acetic anhydride/aq NaOH system was effective to obtain *N*-Aco-*N*-Ac-ABA (22); *N*-Ac-*N*-OH-ABA dissolved in a small amount of 2% aq. NaOH was treated with 1.2 equiv of acetic anhydride at room temperature to give the desired *N*-Aco-*N*-



Scheme 1-1. Synthesis and transformation of *N*-Aco-*N*-Ac-ABA

Ac-ABA as a yellow precipitate. Although the *O*-acetylation of *N*-Ac-*N*-OH-ABA at room temperature smoothly led to *N*-Aco-*N*-Ac-ABA, the same reaction at 0 °C led to an unidentified compound instead of the expected one.

Reaction of N-Acetoxy-N-acetyl-3-aminobenzanthrone (N-Aco-N-Ac-ABA) with dG and Characterization of dG-N-Ac-ABA Adduct

Among several ABA derivatives synthesized in the present work, only *N*-Aco-*N*-Ac-ABA reacted successfully with dG at pH 5 and 7. HPLC analysis of the reaction product at 60 °C showed new peaks, which were not observed in a control reaction without deoxyribonucleoside. The product eluting at 67.4 min on HPLC was collected by reversed phase LPLC and further purified by HPLC. The UV spectrum of this adduct showed the absorption maxima at 280 and 400 nm (Figure 1-1, (a)). The positive ion ES-MS spectrum with on-line HPLC of the purified adduct showed a molecular ion peak at *m/z* 553 $(M+H)^+$ and a daughter ion peak at *m/z* 437 arising from the loss of a deoxyribose moiety from the parent ion, consistent with the adduct formation from *N*-Aco-*N*-Ac-ABA and 2'-deoxyguanosine (Figure 1-1, (b)). The ^1H NMR spectrum in DMSO-d_6 revealed the absorptions of all sugar protons at δ 2.0-5.8 and of N^{1}H and NH_2 groups of the guanine moiety at δ 10.8 and 6.4, respectively (Figure 1-2, Table 1-1). A singlet peak at δ 2.0 was assigned to the acetyl group. A broad ^1H NMR signal at δ 10.2 underwent complete isotope exchange with D_2O in DMSO-d_6, assigning the absorption to the amide functionality. Irradiation of this amide signal showed an NOE effect (-4 %) on H-4 proton of benzanthrone. All these observations are consistent with the location of the acetoamido group at C-3 position

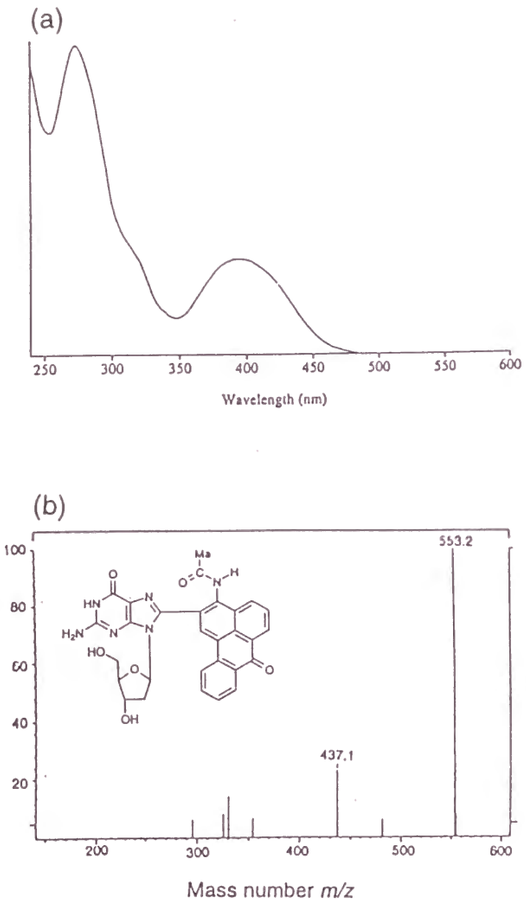


Figure 1-1. (a) On-line HPLC-UV spectrum and (b) ES-MS spectrum of dG-*N*-Ac-ABA

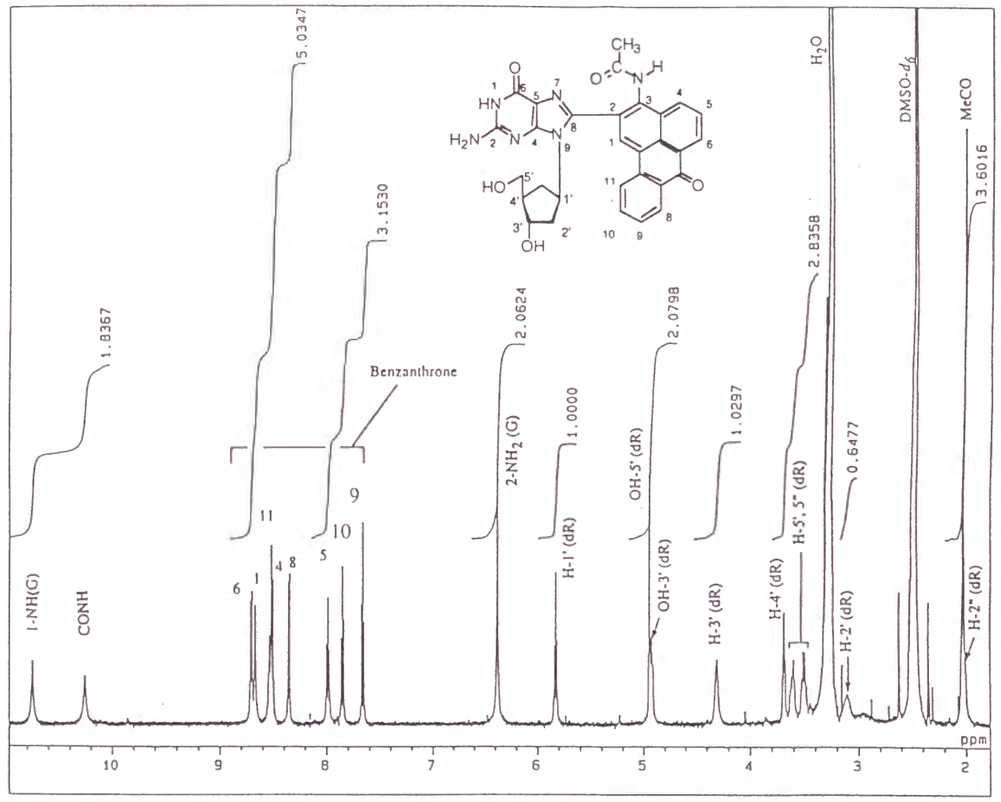


Figure 1-2. 500-MHz ^{1}H-NMR spectrum of of dG-*N*-Ac-ABA in DMSO-d_6

Table 1-1 500 MHz ^{1}H NMR Spectral Parameters of dG-*N*-Ac-ABA

δ (ppm)	multiplicity	assignment
10.73	s	N1H (G)
10.16	s	CONH (amide)
8.71	d	H-6 (BA)
8.67	s	H-1 (BA)
8.54	d	H-4 (BA)
8.51	d	H-11 (BA)
8.36	d	H-8 (BA)
7.99	t	H-5 (BA)
7.86	dt	H-10 (BA)
7.67	t	H-9 (BA)
6.37	s (2)	2-NH_2 (G)
5.84	t(1)	H-1'(dR)
4.95	d	OH-5'(dR)
4.93	t	OH-3'(dR)
4.36	m(br)	H-3'(dR)
3.70	ddd	H-4'(dR)
3.61	ddd	H-5'(dR)[a]
3.51	dd	H-5"(dR)[a]
3.11	m(br)	H-2'(dR)
2.03	s	H-2"(dR) and CH_3 (amide)

(Hz)

$J_{4,5}$= 8.0	$J_{5,6}$=8.0	$J_{8,9}$=7.2	$J_{9,10}$=7.1
$J_{10,11}$=8.0	$J_{1'2'}$=~7.1	$J_{1'2"}$=~6.9	$J_{2'2"}$= ~10.2
$J_{2"3'}$= ~5.3	$J_{2'3'}$= 2.9	$J_{3'4'}$=~3.0	$J_{4'5'}$=4.8
$J_{5'5"}$=11.5	$J_{4'5"}$=5.4	$J_{5'\text{-OH}}$=4.4	$J_{5'\text{-OH}}$=4.5
$J_{3'\text{-OH}}$= 5.0			

[a] Assignment can be reversed.

of benzanthrone nucleus.

The downfield peaks between δ 7.6 and 8.8 corresponded to 8 protons of benzanthrone, and the C-8 proton signal of guanine that normally appears at δ 7.9 was not observed. Furthermore, the characteristic *ortho* coupling pattern of H-1 and H-2 protons in 3-substituted benzanthrone were missing and a sharp singlet appeared at δ 8.68, instead. A possibility that this singlet signal came from the C-8 proton of guanine was ruled out because of its lower chemical shift as compared to those of known nitro-PAH-dG adducts. Thus, the coupling is highly likely to have occurred between the C-2 or C-1 of benzanthrone and C-8 of guanine. The COSY experiment enabled us to assign seven aromatic protons from H-4 to H-11 of benzanthrone moiety. Irradiation of the remaining singlet at δ 8.68 showed an NOE effect (-5%) on the H-11 proton signal, thus allowing the assignment of this signal to H-1 of benzanthrone and excluding the possibility of this singlet peak to arise from H-8 of deoxyguanosine. On the basis of these observations, the author may safely conclude that the benzanthrone was connected at C-2 position to the C-8 position of dG. This structure assignment was further confirmed through ^{13}C NMR spectroscopy, where all 27 carbon atoms could be observed and those bound to a single H were enhanced in distortionless enhancement by polarization transfer (DEPT) experiment (Table 1-2). The absence of ^{13}C signal enhancement of guanine C-8 and benzanthrone C-2 on DEPT endorsed that the C-2 of benzanthrone was bound to the C-8 of guanine. On the basis of these NMR and MS data, the adduct is unanimously assigned as *N*-acetyl-3-amino-2-(2'-deoxyguanosin-8-yl)benzanthrone. For other nitro and amino compounds, the adduct formation with dG generally occurs

at C-8 through the amine or amide group. However, this is not the case with our adduct.

Table 1-2 ^{13}C-NMR Chemical shift of dG-*N*-Ac-ABA

δ (ppm)	assignment	δ (ppm)	assignment
182.01	C7(BA)	127.63	C-11b (BA)
169.05	C=O (amide)	127.13*	C-8 (BA)
156.68	C6 (G)	127.01*	C-5 (BA)
153.16	C2 (G)	126.28*	C-1 (BA)
151.09	C4 (G)	125.56	C-11c (BA)
144.56	C8 (G)	123.82	C-2 (BA)
136.15	C3 (BA)	123.67*	C-11(BA)
134.71	C-11a (BA)	117.29	C-5 (G)
133.88*	C-10 (BA)	87.65*	C-4 (dR)
131.70*	C-4 (BA)	84.91*	C-1 (dR)
129.93*	C-6 (BA)	70.97*	C-3(dR)
129.87	C-7a (BA)	62.13	C-5 (dR)
129.16	C-6a (BA)	36.95	C-2 (dR)
128.60*	C-9 (BA)	22.71*	CH_3 (amide)
127.65	C-3a (BA)		

Assignments are made on 2D-NMR experiments as well as by direct comparison with 2'-deoxygunosine and benzanthrone [27].
Peaks enhanced in DEPT are marked with an asterisk.

Reaction of N-Acetoxy-N-acetyl-3-aminobenzanthrone with Calf Thymus DNA

Calf thymus DNA was reacted with *N*-Aco-*N*-Ac-ABA at 50 ˚C for 15 h. Hydrolysis of the product with enzymes followed by HPLC analysis showed an adduct at exactly the same retention time as that of dG-*N*-Ac-ABA adduct (Figure 1-3). The adduct appearing at 67.37 min coeluted with the authentic dG-*N*-Ac-ABA adduct and showed the identical on-line UV spectrum. Thus, the author has confirmed that the NBA-dG adduct was also formed from the reaction of *N*-Aco-*N*-Ac-ABA and calf thymus DNA.

In the HPLC chromatogram of the DNA hydrolysate, several additional peaks were observed. Since unchanged four nucleosides were all eluted within 20 min, compounds appearing after 20 min may represent other DNA adducts of *N*-Aco-*N*-Ac-ABA. HPLC-MS analysis of a peak at 59.80 min showed a molecular ion of *m/z* 537, corresponding to a dA-*N*-Ac-ABA adduct. The peak at 59.80 min was also detected in the HPLC chromatogram of the reaction mixture from dA and *N*-Aco-*N*-Ac-ABA, suggesting the adduct formation between dA and *N*-Aco-*N*-Ac-ABA. However, the other peaks could not be solved yet.

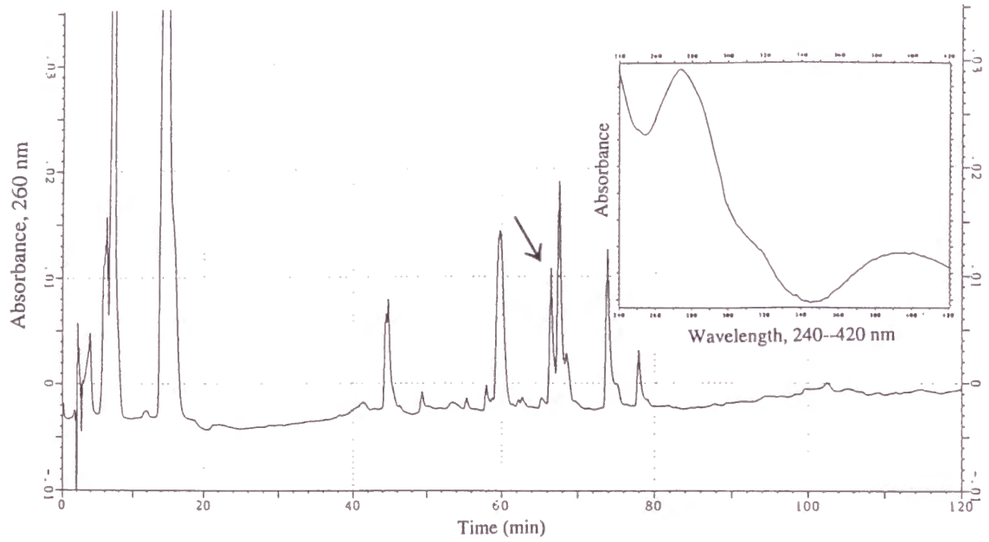


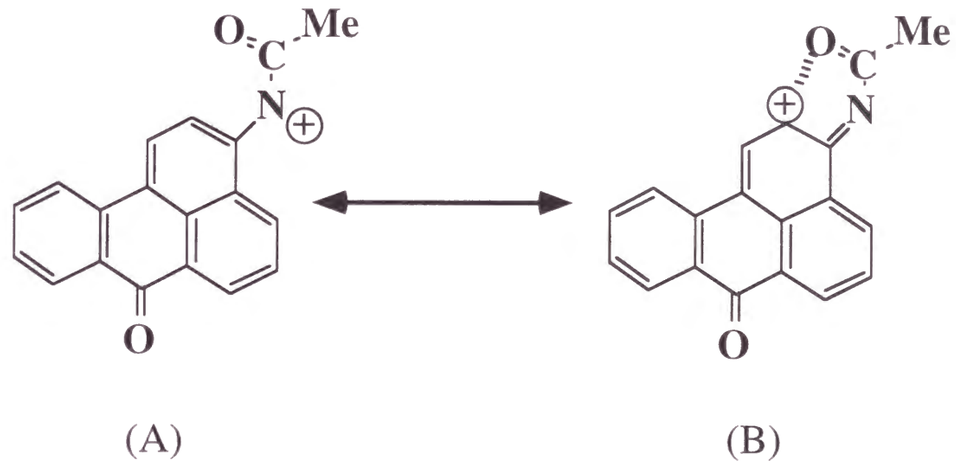
Figure 1-3. HPLC analysis and on-line UV spectra of the enzymatic digest of calf thymus DNA modified with *N*-Aco-*N*-Ac-ABA

Discussion

In this research, the author has synthesized *N*-acetoxy-*N*-acetyl-3-aminobenza nthrone (*N*-Aco-*N*-Ac-ABA) and made it to react with dG and calf thymus DNA, obtaining the dG-*N*-Ac-ABA adduct in a yield of around 10%. Synthesis and reactions of the DNA and dG adducts from *N*-acetoxy-*N*-acetylamino derivatives of fluorene and biphenyl compounds have already been studied well. In those studies, *N*-acetoxy-*N*-acetyl-2-aminofluorene was found to react with DNA and dG to give several dG-adducts in good yield (24). However, the reaction of *N*-acetoxy-*N*-acetyl-4-aminobiphenyl with dG failed to produce similar dG-adducts due to facile hydrolytic cleavage of the *N*-acetoxy group, which makes it difficult to generate a nitrenium ion species (30). Underwood *et al.* showed that *N*-acetyl-*N*-(2,6-dichlorobenzoyloxy)-4-aminobiphenyl was reluctant to hydrolysis and reacted with dG to give the corresponding adduct in moderate to good yield (23). In the case of this research, however, replacement of the acetoxy group in *N*-Aco-*N*-Ac-ABA by the 2,6-dichlorobenzoyloxy group was not fruitful for the adduct formation. *N*-Acetyl-*N*-(2,6-dichlorobenzoyloxy)-3-aminobenzanthrone was found to be so stable that it neither reacted with dG nor went to hydrolysis even at temperatures as high as 100 °C. Acetyl salicylate was reported to be useful for the synthesis of dG-C8-aminofluo rene adduct (31), but it did not work satisfactorily on *N*-OH-ABA to give the dG adduct. *N*-OH-ABA was mixed with a plasmid DNA at pH 5 in ethanol/sodium citrate buffer, but the author could not observe any increase in the frequency of mutation in *supF* shuttle vector system (32). This finding suggests that the DNA adduct formation by *N*-OH-ABA does not take place effectively. Although direct

acetoxylation of the hydroxylamino function using acetyl cyanide was reported (17-19), the author has not yet examined it.

The dG adduct obtained in this study has been shown to possess a unique chemical structure, quite different from those derived from other aromatic nitro or amino compounds as previously reported. The adduct formation of nitro-PAH metabolites generally occurs at C-8 of dG through the amino or amide group, or at N^2 of dG through the most cationic ring carbon resulting from the resonance-stabilizatio n of the initial nitrenium ion intermediate (8,9,29). For nitropyrenes, nitrofluoranthenes, and nitrobiphenyls, the C-8 substituted dG adducts are known to make significant contribution to the total DNA adducts (7,25-28). For nitro-PAH of greater ring numbers, such as 3-nitrobenzo[*a*]pyrene and 6-nitrochrysene, the N^2-substituted guanine adducts are formed to considerable extent owing to the increased contribution of the carbocationic resonance form of a putative nitrenium ion intermediate (7, 19). In the case of NBA, however, the coupling occurred at C-8 of dG through C-2 of benzanthrone. This type of C-C bond coupling in the reaction of dG with aromatic *N*-acetoxy-amino compounds has not previously been reported, though there are a few scattered papers that report the C-C bond formation of electronically oxidized benzopyrene and dibenzocarbazole with dG or dA (33-35). The new type of adduct obtained in this research would plausibly be formed via the isomerization of the initially formed nitrenium ion (Scheme 1-2, (A)) to an energetically favored arenium ion (Scheme 1-2, (B)) with a localized positive charge at C-2 position, followed by attack of this carbocation on C-8 position of dG. However, it is also possible for this carbocation to attack the N^2 position of dG to



Scheme 1-2. Resonance-stabilization of a putative nitrenium ion intermediates from *N*-Aco-*N*-Ac-ABA

give an N^2-adduct. HPLC-MS analysis of the product mixture from the reaction of *N*-Aco-*N*-Ac-ABA with dG showed the presence of an additional adduct of a molecular ion peak at *m/z* 553, but its amount was too small to identify the product.

The conformation of the glycoside linkage appears to play an important role in adduct persistence *in vivo* and consequently it may influence the toxicological properties of DNA adducts (29,36-41). The *syn* type adduct appears to induce a greater distortion of the DNA helix than the *anti* type one, resulting in rapid disappearance of the former adduct and greater persistence of the latter. Chemical shift of the sugar H-2' is generally a good probe for the preferred conformation of the glycoside linkage in dG, appearing at δ 2.5 ~ 3.1 due to the deshielding effect of nearby guanine N-3 atom in the *syn* conformation (36-41). The H-2' atom of dG-*N*-Ac-ABA adduct was found to absorb at δ 3.1, which was considerably downfield as compared with H-2' of the parent dG (δ 2.5), suggesting the conformation of this adduct to be *syn*. Upfield shift of the sugar C-2' absorption at δ 36.9 of dG-*N*-Ac-ABA in ^{13}C NMR spectrum (generally δ 40) also supports this conformation (36-41).

Although the metabolic activation and *in vivo* DNA adduct formation of NBA have not been studied yet, alike other nitro arenes the *N*-hydroxy derivatives of ABA as well as the ultimate mutagenic species like *N*-acetoxy, *N*-sulfonyloxy, or *N*-propyloxy derivatives of ABA are all highly likely to be probable mutagenic metabolites of NBA. In order to examine whether they form the unique C-2 adduct like dG-*N*-Aco-ABA or ordinary C-8 and N^2-adducts *in vivo*, further study is ongoing.

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CHAPTER 2

A ^{32}P-postlabeling analysis of DNA adducts formed in human hepatoma HepG2 cells treated with 3-nitrobenzanthrone

Abstract

3-Nitrobenzanthrone (NBA) is a mutagenic aromatic compound which is found in diesel exhaust and airborne particles. In this Chapter, the ^{32}P-postlabeling analysis was used to examine the adducts in DNA from human hepatoma HepG2 cells treated with NBA. Two major and two minor adduct spots were obtained in the analysis. The structure of the compound in an obtained minor adduct spot was identified to be *N*-acetyl-3-amino-2-(2'-deoxyguanosin-3',5'-bisphosphate-8-yl)-benzanthrone, based on TLC mobility of the compound in comparison with that of synthetic standard.

Introduction

3-Nitrobenzanthrone (NBA, 3-nitro-7*H*-benz[*d,e*]anthracen-7-one) is a powerful mutagenic aromatic nitroketone which is found in diesel exhaust and airborne particles (1). Its mutagenicity by Ames *Salmonella typhimurium* (TA98) assay is as high as that of 1,8-dinitropyrene (1,8-DNP) which is the strongest mutagen so far reported (1). The compound also induces micronuclei in mouse peripheral blood reticulocytes and, therefore, is thought to be genotoxic to mammals and a suspected human carcinogen (1).

Metabolic activation of nitroaromatic hydrocarbons occurs through *N*-reduction to form the *N*-hydroxy arylamine, which can bind to DNA, or may undergo further activation by esterification to produce highly reactive species which react with DNA (2-5). The covalent binding of genotoxic mutagens to DNA is regarded as a critical event in cancer initiation. In the case of NBA, reaction of a chemically synthesized reactive form, *N*-acetoxy-*N*-acetyl-3-aminobenzanthrone (*N*-Aco-*N*-Ac-ABA), with calf thymus DNA results in the formation of DNA adducts: *N*-acetyl-3-amino-2-(2'-deoxyguanosin-8-yl)-benzanthrone (dG-*N*-Ac-ABA) and a structure unidentified dA adduct (dA-*N*-Ac-ABA) *in vitro* (Chapter 1)(6). The dG-*N*-Ac-ABA possesses a unique chemical structure, that is quite different from those derived from other aromatic nitro or amino compounds as previously reported. The adduct formation of nitro-PAH metabolites generally occurs at C-8 of dG through the amino or amide group, or at N^2 of dG through the most cationic ring carbon resulting from the resonance-stabilization of the initial nitrenium ion intermediate (2,7). In the case of dG-*N*-Ac-ABA, the coupling between nucleoside and the aromatic compound

occurred at C-8 of dG through C-2 of benzanthrone. This type of C-C bond coupling in the reaction of dG with aromatic *N*-acetoxy-amino compounds has not previously been reported. In this study, DNA samples from human hepatoma HepG2 cells treated with NBA were analyzed by ^{32}P-postlabeling to examine the formation of these adducts in the cells.

Materials and methods

Materials

NBA and *N*-acetyl-*N*-hydroxy-3-aminobenzanthrone (*N*-Ac-*N*-OH-ABA) were synthesized as described (Chapter 1)(6). RNase T1, micrococcal nuclease and spleen phosphodiesterase were purchased from Worthington Biochemical Co. (Freehold, NJ). Nuclease P1 and T4 Polynucleotide kinase were obtained from Yamasa Shoyu Co. (Chosi, Japan) and Toyobo Biochemicals (Osaka, Japan), respectively. [γ-^{32}P] ATP (6000 Ci / mmol) was purchased from Amersham Pharmacia Biotech (Tokyo, Japan). Proteinase K, phosphodiesterase I (Type VII), RNase A (Type IA), 2'-deoxyguanosine-3'-monophosphate (3'-dGp) and 2'-deoxyadenosine-3'-monophosphate (3'-dAp) were obtained from Sigma (St. Louis, MO). Polyethyleneimine(PEI)-cellulose sheet (Polygram Cell 300 PEI) was purchased from Machery-Nagel (Düren, Germany). HPLC analysis was performed on a Waters 600E HPLC system equipped with a Waters 991J photodiode array UV detector. Radioactivity was measured by Packared liquid scintillation analyzer TRICARB 2300TR.

N-Acetyl-3-amino-2-(2'-deoxyguanosine-3'-monophosphate-8-yl)benzanthrone (3'-dGp-N-Ac-ABA) and dAp-N-Ac-ABA

N-Ac-*N*-OH-ABA (1 mg) was dissolved in 0.05 mL of 0.5 N NaOH. After 0.8 µL of acetic anhydride was added to the solution, the precipitate was filtered and washed twice with 1 mL water. *N*-Aco-*N*-Ac-ABA was obtained as a yellow solid. This solid was used without further purification for the reaction with 3'-dGp or

3'-dAp. The solid was suspended in 0.1 mL of acetonitrile and incubated with 1 mg of 3'-dGp or 3'-dAp (10 mg/mL in 10 mM sodium citrate, pH 5, each) at 55 °C for overnight. The reaction mixture was subjected to HPLC using a reverse-phase column of Waters µbondosphere C_{18} (3.9 mm x 15 mm), eluted over 45 min at a flow rate of 1.0 mL/min with linear gradient of 0.05 M triethylammonium acetate (TEAA), pH 7.0, containing 1-50% acetonitrile. The fractions containing the adduct were evaporated to dryness and the residue was redissolved in DMSO.

Cell culture

A human hepatoma HepG2 cell line was obtained from RIKEN Cell Bank (Wako, Japan). The cells were cultured in Dulbecco's modified minimum essential medium (D-MEM; Nikken, Kyoto, Japan) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). Monolayer cultures of HepG2 cells were initiated at a density of 1 x 10^6 cells / 100 mm tissue culture dish. After overnight incubation, the cultured cells (80% confluent) were washed with Dulbecco's phosphate buffered saline (PBS) solution (pH 7.5), and 10 mL of D-MEM were added to each dish. NBA, dissolved in 100 µL of dimethylsulfoxide (DMSO), was added to each dish at the concentrations shown in Table 2-1, and the culture incubated an additional 8 hours. The treated cells were then dispersed with trypsin, and the DNA was extracted as described below.

The cytotoxicity of NBA was determined by neutral red-staining in a 96-well microplate. The cells (80% confluent per well) were treated with NBA for 8 hours, then stained and fixed, and A_{540} was measured by a microplate reader.

DNA isolation

Cells were resuspended in 3 mL of 150 mM NaCl, 10 mM Tris-HCl (pH 8.0) and 10 mM EDTA. After addition of 30 µL of 10% SDS, the cell suspension was incubated at 37 °C for 1 hour with Proteinase K (100 µg / mL). Successive extractions were performed with 1 volume each of phenol (saturated with 0.1 M Tris-HCl, pH 8), a 1:1 mixture of phenol/sevag (chloroform / isoamyl alcohol 24:1) and sevag by inverting the tube 100 times. After addition of 0.1 volume of 5 M NaCl, DNA was precipitated by addition of 1 volume of ethanol precooled at -20 °C and centrifugation. The DNA was washed twice with 70% ethanol and dissolved in 0.5 mL 0.01 x SSC / 1 mM EDTA (1 x SSC = 0.15 M NaCl / 0.015 M sodium citrate). To remove the impurities of the DNA samples, the DNA solution was treated with RNase A (100 µg / mL) and RNase T1 (33 u / mL) for 1 hour at 37 °C followed by Proteinase K (20 µg / mL), and the above organic solvent extraction procedure was repeated. The concentration of the DNA was estimated spectrophotometrically (A_{260}) and adjusted to 1 mg / mL.

^{32}P-Postlabeling analysis

A ^{32}P-postlabeling analysis was performed following the methods reported previously with a little modification (8-10). Briefly, each DNA (15 µg) was digested to 3'-monophosphate nucleotides by micrococcal nuclease and spleen phosphodiesterase at 37 °C for 6 hours. Then adduct enrichment was carried out with nuclease P1 at 37 °C for 1 hour. The digest was evaporated to dryness and labelled with ^{32}P by using T4 polynucleotide kinase and [γ-^{32}P]ATP. The samples

were analyzed by following two conditions:

(i) The ^{32}P-labeled 3',5'-bisphosphate nucleotide (3',5'-[^{32}P]pdNp) sample was spotted on a 10 x 10 cm polyethyleneimine (PEI)-cellulose thin-layer chromatograph (TLC) plate at a location 1 cm from the bottom and 1 cm from left edge of the plate and developed using four different solvents. The plate was washed twice with distilled water for 30 min at room temperature and air dried. The plate were developed for 16 hours in 2.3 M sodium phosphate buffer (pH 6.0) with a paper wick (D1), and subsequently developed in the same direction to the top of the plate with 1.5 M lithium formate and 3 M urea (pH 3.5) (D2). Then the plate was developed at a right angle to the previous direction with 0.6 M LiCl, 0.25 M Tris-HCl, and 3 M urea (pH 8.0) (D3). The plate was subsequently developed in the same direction with 2.3 M sodium phosphate buffer (pH 6.0) to 5 cm onto paper wick (D4). The position of adducts was visualized by autoradiography, using Amersham Hyper film MP. For fine analysis of spots around the origin on TLC plate, the plate was further developed with following solvent system: 2 M lithium formate and 6.5 M urea (pH 3.5) from bottom to top (same direction as D1, 2) and 0.6 M LiCl, 0.25 M Tris-HCl, and 6.5 M urea (pH 8.0) from left to right (same direction as D3, 4) followed by 2.3 M sodium phosphate buffer (pH 6.0) from left to right with 5 cm paper wick. Relative adduct labeling (RAL) was determined by the method of Gupta (9).

(ii) The ^{32}P-labeled 3',5'-bisphosphate nucleotide sample was treated with phosphodiesterase I (PDE I) at 37 °C for 1 hour (11). The digested 5'-[^{32}P]monophosphate nucleotide (5'-[^{32}P]pdN) sample was separated by the same condition described in (i).

Analysis of DNA adduct by HPLC

The ^{32}P-labeled sample with an additional treatment using PDE I was developed on TLC plate. The adduct spot corresponding to 5'-[^{32}P]pdG-*N*-Ac-ABA on TLC plate was cut out and extracted with 0.5 mL of 4 M pyridinium formate (pH 4.5) solution twice with shaking for 30 min. The mixture was centrifuged, and supernatant was collected by passing through a 0.2 μm filter and evaporated. The authentic sample of 5'-pdG-*N*-Ac-ABA was synthesized by the same procedure for 3'-dGp-*N*-Ac-ABA as described above. An aliquot of the radioactive sample was mixed with appropriate amount of the authentic sample and subjected to HPLC on a Waters μbondosphere C_{18} column (3.9 mm x 15 mm) with the following solvent system at a flow rate 1.0 mL / min at 40 °C: 0 - 10 min, 10% acetonitrile in 0.05 M TEAA (pH 7.0), 10 - 45 min, a linear gradient of 10 - 50 % acetonitrile in 0.05 M TEAA (pH 7.0), 45 - 50 min, 50 % acetonitrile in 0.05 M TEAA (pH 7.0). The eluent was monitored by measuring its absorbance at 395 nm and collected at 2 min intervals. Radioactivity of each fraction was measured by a liquid scintillation analyzer.

Results and discussion

The ^{32}P-postlabeling TLC profiles of synthetic 3',5'-[^{32}P]pdGp-*N*-Ac-ABA and 3',5'-[^{32}P]pdAp-*N*-Ac-ABA adduct standards, DNA from untreated human hepatoma HepG2 cells, DNA from the cells treated with 9 μM NBA for 8 hours, DNA from treated the cells spiked with the adduct standards are shown in Figure 2-1. Spots co-migrating with the adduct standards 3',5'-[^{32}P]pdGp-*N*-Ac-ABA and 3',5'-[^{32}P]pdAp-*N*-Ac-ABA appeared after DNA from the cells treated with NBA was developed (Figure 2-1 C, D). No spot was observed after DNA from non-treated cells was developed (Figure 2-1 E).

Several other spots were also observed around the origin on TLC plate after DNA from treated cells was developed. For analysis of these spots, further development were performed using solvents with higher salt and urea concentrations (Figure 2-2). The spots consisted of two spots (Spot 3, Spot 4). As in the case of PhIP-DNA adducts (11), spots 3 and 4 might be undigested dinucleotides or oligonucleotides containing the dG-adduct or the dA-adduct. Therefore, the author examined a ^{32}P-labeled sample further treated with phosphodiesterase I (PDE I), as for PhIP- DNA adducts (11). However, the number of spots and relative positions of migration were not influenced (data not shown). This result might indicate that spots 3 and 4 were not undigested nucleotides.

A HPLC analysis was performed to reinforce the identification of 5'-pdG-*N*-Ac-ABA. The ^{32}P-labeled sample with an additional PDE I treatment was developed on TLC plate. The spot corresponding to 5'-[^{32}P]pdG-*N*-Ac-ABA on TLC plate was extracted with pyridinium formate and subsequently subjected to the

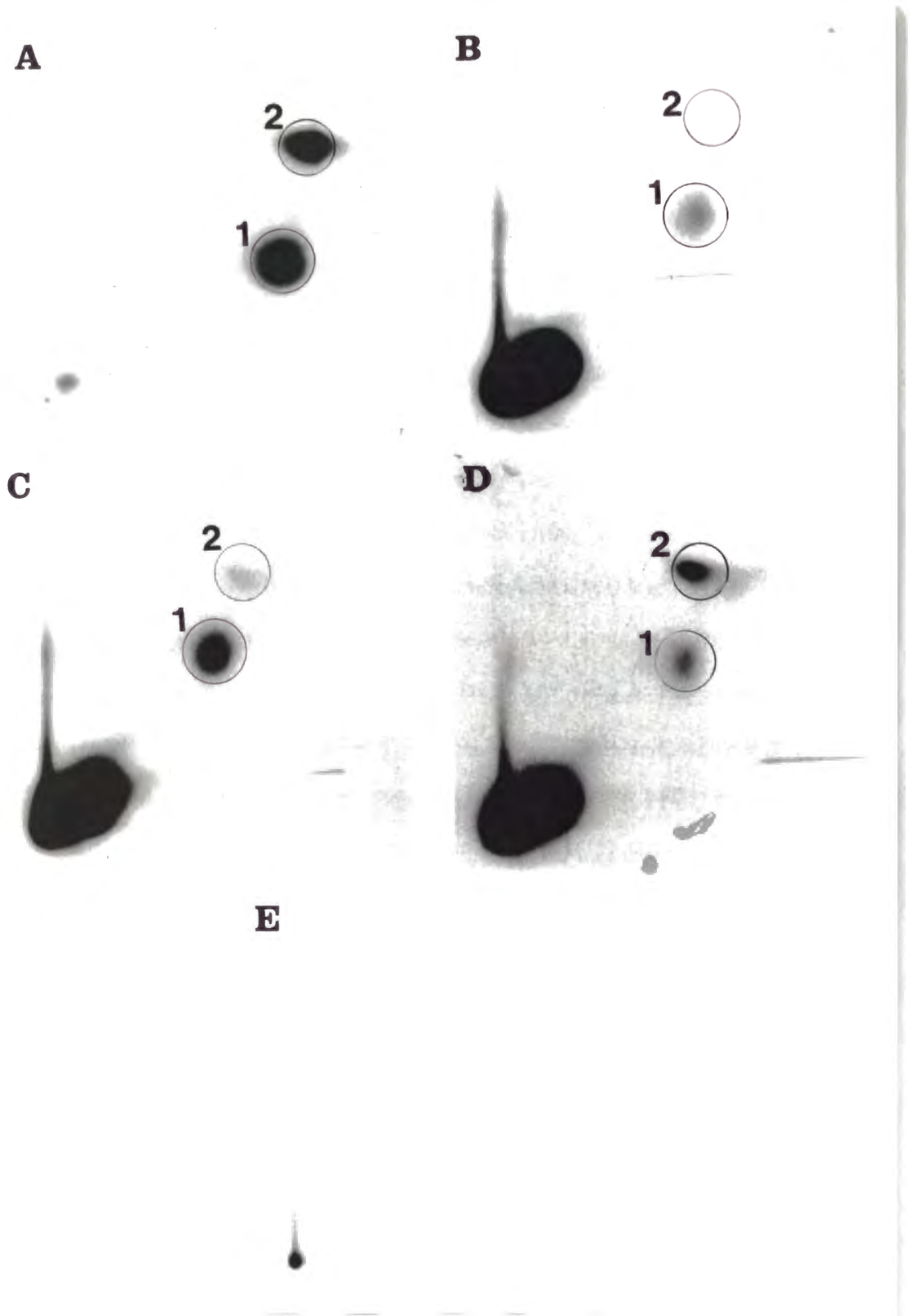

Figure 2-1. ^{32}P-Postlabeling analysis of (A) standard DNA adducts, 1: 3',5'-[^{32}P]pdGp-*N*-Ac-ABA, 2: 3',5'-[^{32}P]pdAp-*N*-Ac-ABA; (B) DNA from an NBA treated cells (9 μM for 8 hours); (C) DNA from NBA treated cells spiked with the adduct standard 3',5'-[^{32}P]pdGp-*N*-Ac-ABA; (D) DNA from NBA treated cells spiked with the adduct standard 3',5'-[^{32}P]pdAp-*N*-Ac-ABA; (E) DNA of control cells.

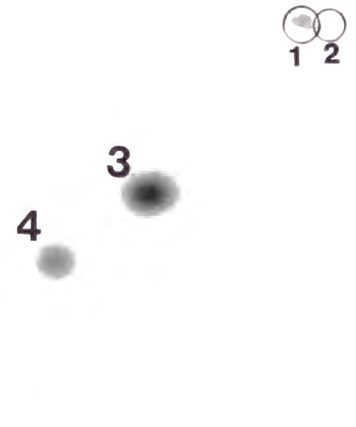

Figure 2-2. ^{32}P-Postlabeling analysis for development of spots observed around the origin in Figure 2-1. The TLC plate of Figure 2-1 (B) was further developed using higher salt and urea concentration solvents. Time of exposure to X-ray film was shorter than that of Figure 2-1.

HPLC analysis with the unlabeled synthetic 5'-pdG-*N*-Ac-ABA adduct standard as a visual marker. The elution position of the major radioactivity on HPLC coincided with that of the UV absorption peak of authentic 5'-pdG-*N*-Ac-ABA at retention time 31-33 min as shown in Figure 2-3. The HPLC analysis of 5'-pdA-*N*-Ac-ABA could not be performed because of weak radioactivity of the spot corresponding to 5'-[^{32}P]pdA-*N*-Ac-ABA.

Levels of adducts in the cells treated with NBA for 8 hours are shown in Table 2-1. Levels of all four spots increased with an increase in concentration of NBA. Spots 3 and 4 were the predominant adducts. Levels of 3',5'-[^{32}P]pdGp-*N*-Ac-ABA and 3',5'-[^{32}P]pdAp-*N*-Ac-ABA were less than 1/100 of those of spots 3 and 4.

In this study, the author found that four adducts (two major adducts and two minor adducts) exist in the DNA from the cells treated with NBA. Two minor adducts were identified as 3',5'-pdGp-*N*-Ac-ABA and 3',5'-pdAp-*N*-Ac-ABA, based on TLC mobility in comparison with synthetic standards. However, the structures of major DNA adducts are still unknown. Of known adducts between DNA and aromatic amines or nitro-PAH, the adducts with the *N*-acetyl group are generally found as minor adducts and those without *N*-acetyl group are found as major adducts *in vivo* (7). Therefore, spot 3 and 4 might be adducts without *N*-acetyl group. Further study should be done.

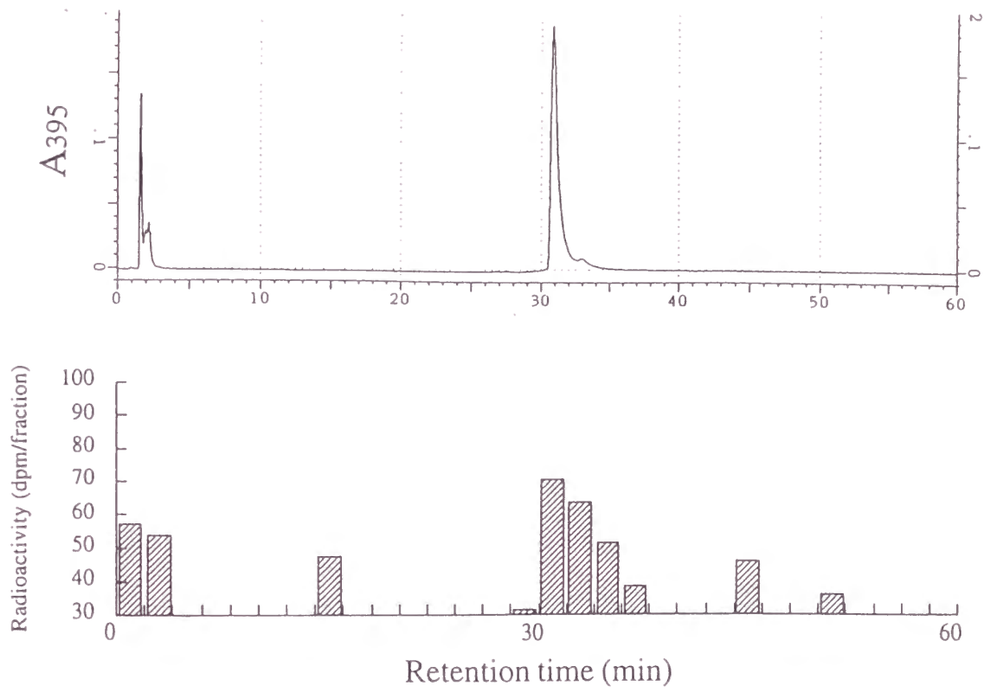

Figure 2-3. HPLC elution profiles of a mixture containing an extract of the adduct spot corresponding to 5'-[^{32}P]pdG-*N*-Ac-ABA with a standard DNA adduct 5'-pdG-*N*-Ac-ABA. The ^{32}P-labeled sample with an additional treatment using PDE I was developed on TLC plate. After extraction of the adduct spot, the extract was subjected to HPLC analysis.

Table 2-1 DNA adduct levels in human hepatoma HepG2 cells treated with NBA

		DNA adduct levels (RAL[a] x 10^8)			
NBA (μM)	Cell viability (%)	Spot 1: 3',5'-[^{32}P]pdGp-*N*-Ac-ABA	Spot 2: 3',5'-[^{32}P]pdAp-*N*-Ac-ABA	Spot 3	Spot 4
0	100	N.D.	N.D.	N.D.	N.D.
0.9	67±1.8	0.95±0.32	0.87±0.18	106±3.3	185±3.1
9	56±3.5	2.8±0.59	1.2±0.17	254±29	364±14

The values are mean ± SD of at least three assays.

N.D.: not detected (RAL < 1 x 10^{-9})

[a]RAL: relative adduct labeling

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CHAPTER 3

Mutagenic specificity of a derivative of 3-nitrobenzanthrone in the *supF* shuttle vector plasmids

Abstract

3-Nitrobenzanthrone (NBA) is a powerful bacterial mutagen and a suspected human carcinogen present in diesel exhaust and airborne particulates (1). In the Chapter 1 (2), *N*-acetoxy-*N*-acetyl-3-aminobenzanthrone (*N*-Aco-*N*-Ac-ABA) was synthesized to yield the DNA adducts of NBA. In this Chapter, to investigate the mutagenic specificity of NBA in human cells, the author analyzed mutations induced by *N*-Aco-*N*-Ac-ABA using the *supF* shuttle vector plasmids. Base sequence analysis of 110 and 100 plasmids with mutations in the *supF* gene propagated in normal cells (WI38-VA13) and nucleotide excision repair deficient cells (XP2OS (SV)), respectively, revealed that the majority of the mutations was base substitutions (85 % and 90 %) and the rest were deletions and insertions (10 % and 15 %) in both cell lines. About half of the mutant plasmids had a single base substitution. Of the base substitutions, the most frequent mutation was G:C to T:A transversion (41 % and 51 %), followed by G:C to A:T transitions (18 % and 24 %) in

either cell. The mutations were distributed not randomly but located at several hot spots, and almost all (9 of 10) hot spots were at the sites of G:C base pairs. The polymerase-stop assay in the *supF* gene revealed that *N*-Aco-*N*-Ac-ABA preferentially bound to guanine residues, and mutation sites were generally consistent to the sites where the guanine adducts were formed.

Introduction

3-Nitrobenzanthrone (NBA[1], 3-nitro-7*H*-benz[*d,e*]anthrace-7-one) is a powerful mutagenic aromatic nitroketone present in diesel exhaust and airborne particles (1). Mutagenicity of NBA by the Ames *Salmonella typhimurium* (TA98) assay is as high as that of 1,8-dinitropyrene (1,8-DNP), the strongest mutagen so far reported (1). The compound also induces micronuclei in mouse peripheral blood reticulocytes and, therefore, is thought to be genotoxic to mammals and a suspected to be a human carcinogen (1).

Most carcinogens are metabolically activated in cells and bind covalently to DNA (3). Nitroaromatic hydrocarbons need to be metabolized into reactive electrophiles to exert their carcinogenic activity (4). The initial activation of nitroaromatic hydrocarbons is through the reduction catalyzed by both microsomal and cytosolic enzymes to form *N*-hydroxy arylamines (4). *N*-Hydroxy arylamines are further metabolized to *N*-hydroxy arylamids, *N*-sulfonyloxy arylamines, *N*-acetoxy arylamines and *N*-sulfonyloxy arylamids, by several transferases (4)(5). These esters except for *N*-hydroxy arylamids undergo heterolysis of the N-O bond to produce strongly electrophilic arylnitrenium / carbonium ions and react with DNA to form adducts (6)(7). In the Chapter 1 (2), the author synthesized an *N*-acetoxy-*N*-acetyl derivative of NBA, *N*-acetoxy-*N*-acetyl-3-aminobenzanthrone (*N*-Aco-*N*-Ac-ABA), which produces reactive arylnitrenium / carbonium ions of NBA and showed that this derivative reacted with DNA and formed the adduct *N*-acetyl-3-amino-2-(2'-deoxyguanosine-8-yl)-benzanthrone (dG-*N*-Ac-ABA) *in vitro*. The author has also confirmed the presence of dG-*N*-Ac-ABA in DNA in human

hepatoma HepG2 cells treated with NBA by the ^{32}P-postlabeling analysis (Chapter 2).

Shuttle vector plasmids have been used to examine the mutations caused by environmental mutagens and carcinogens (8). Mutations induced in the plasmids by mutagens reflect mutations in endogenous genes of mammalian cells in which plasmids are propagated (9). In this study, the author adopted the pMY189 shuttle vector plasmids to analyze the mutational specificity of NBA in human cells. The plasmids were treated with *N*-Aco-*N*-Ac-ABA, and frequencies and types of mutations in the *supF* gene of the plasmids induced by the derivative in normal and repair-deficient human cells were investigated.

Materials and methods

Chemicals

N-Aco-*N*-Ac-ABA was synthesized and purified on column chromatography as described (Chapter 1)(2). Ampicillin, chloramphenicol, nalidixic acid, isopropyl-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) were obtained from Wako Chemicals (Osaka, Japan). Restriction endonuclease *Dpn*I and T4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA) and Toyobo Biochemicals (Tokyo, Japan), respectively. Primer 1 (pBR322 *Eco*RI site primer: 5'-GTATCACGAGGCCCTT-3') was obtained from Takara Shuzo Biochemicals (Tokyo, Japan). Primer 2 (5'-TTCTACGGGGTCTGACGC-3') were synthesized by Nippon Seihun Co. (Tokyo, Japan).

Cells

An SV40-transformed normal human fibroblast cell line WI38-VA13 (10) was obtained from the American Type Culture Collection (Rockville, MD). DNA repair deficient XP2OS(SV) cells were established from a Japanese group A xeroderma pigmentosum (XP) patient (11). All cells were cultured in Dulbecco's modified minimum essential medium (Nikken, Kyoto, Japan) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT).

Shuttle vector plasmid and Bacterial strains

The shuttle vector plasmid pMY189, constructed by Matsuda *et al*. (12), was

used for analysis of mutations. The pMY189 was derived from the pZ189 (8) as described previously (12). The indicator *E.coli* strain KS40/pKY241 (13) is a nalidixic acid-resistant (*gyrA*) derivative of MBM7070 (*lacZ* (am) *CA7070 lacY1 HsdR HsdM* Δ *(araABC-leu)7679 galU galK rpsL thi*)(14), which is used for detection of the mutated *supF* of pMY189. The plasmid pKY241 was constructed by Akasaka *et al.* (13) and contains a chloramphenicol resistant marker and *gyrA* (amber) genes. *E.coli* KS40/pKY241 cells carrying the active *supF* gene are sensitive to nalidixic acid, whereas the cells carrying the mutated *supF* form colonies on the selection plates containing nalidixic acid, chloramphenicol and ampicillin. *E.coli* cells containing wild type *supF* gene produce blue colonies, whereas cells having the mutated *supF* gene produce white or light blue colonies on the selection plates.

Treatment of plasmids with N-Aco-N-Ac-ABA, transfection to human cells, and plasmid recovery

Purified stocks of pMY189 were prepared by using the QIAGEN plasmid purification kit (QIAGEN GmbH, Hilden, Germany). Freshly prepared ethanol solution of *N*-Aco-*N*-Ac-ABA was added to the plasmid suspension (120 μg/mL in 10 mM sodium citrate buffer, pH 6.7). The reaction was allowed to proceed for 1 hour at 37 °C followed by phenol/chloroform extractions and ethanol precipitation of the plasmids to remove the non-reacted excess compound, and the plasmids were redissolved in 800 μL of Dulbecco's phosphate buffered saline (PBS) solution (pH 7.5).

The human cells (2×10^7), WI38-VA13 or XP2OS(SV), and 10 μg *N*-Aco-*N*-Ac-ABA-treated pMY189 in PBS solution (200 μL) were placed in an electroporation chamber (electrodes 0.3 cm apart) (PDS, Inc., Madison, WI) and the cells were transfected with the plasmids by electric pulses (600 V, 4 times). The cells were plated in 10-cm dishes and incubated at 37 °C for 72 hours in a CO_2 incubator. Then plasmids were extracted from the cells using the QIAprep-spin Plasmid kit (QIAGEN GmbH, Hilden, Germany). The purified plasmids were digested with the restriction endonuclease *Dpn*I to eliminate non-replicated plasmids which retain the bacterial methylation pattern.

Selection of mutated supF, and determination of DNA base sequences

Plasmid DNA replicated in the human cells was introduced into the indicator bacterium KS40/pKY241 by the electroporation apparatus *E.coli* Pulsar (Bio-Rad Laboratories, Hercules, CA). The bacterial cells were plated on Luria-Bertani (LB) agar containing 50 μg/mL nalidixic acid, 150 μg/mL ampicillin and 30 μg/mL chloramphenicol, IPTG and X-gal to select the plasmids containing the mutated *supF* genes. A portion of the cells was plated on LB agar containing ampicillin and chloramphenicol to measure the total number of transformants. After the plates were incubated for 24 hours at 37 °C, colonies were counted and mutation frequencies were calculated.

After *E. coli* having mutated plasmids were cultured overnight, the plasmids were extracted and purified with the QIAprep-spin Plasmid kit (QIAGEN GmbH, Hilden, Germany) and the size of the mutated plasmid was checked by agarose gel

electrophoresis. The base sequences of the *supF* gene of the plasmids which were not aberrant in size were determined with the ABI PRISM™ Dye Primer (-21M13) Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Corporation, Foster City, CA) using the 370A automatic DNA sequencer (Perkin-Elmer Corporation, Foster City, CA). The mutants having the identical base changes derived from the same transfection plate were not scored to exclude clusters of the same clones.

The χ^2-goodness of fit test was used to determine if the *N*-Aco-*N*-Ac-ABA-induced base substitution mutations were distributed randomly or non-randomly over the coding region of the *supF* tRNA gene.

Polymerase-stop assay of N-Aco-N-Ac-ABA-treated template

Positions of DNA adducts in the *supF* gene of *N*-Aco-*N*-Ac-ABA-treated plasmids was determined by the *in vitro* DNA polymerase-stop assay (15) as described previously (16)(17). Briefly, double-stranded pMY189 plasmids treated with *N*-Aco-*N*-Ac-ABA (0, 0.2, 2 and 4 mM, at 37 °C for 1 hour in 10 mM sodium citrate buffer, pH 6.7) were denatured and annealed with ^{32}P end-labeled primer 1 (complementary to the sequence of non-coding strand at sites from -46 to -31 in the pMY189) or primer 2 (complementary to the sequence of coding strand at sites from 211 to 228 in the plasmid). Primers were labeled with [γ-^{32}P] ATP (3000 Ci/mmol) using T4 polynucleotide kinase. The polymerization reaction was performed in a manner similar to the sequencing reactions using a Tth Polymerase DNA Sequencing kit "Sequencing PRO" (Toyobo Biochemicals, Tokyo, Japan), except that dideoxynucleotides were omitted, and DNA adducts were allowed to terminate

polymerization. DNA from the four dideoxy sequencing reactions with an untreated template and a ^{32}P-labeled primer was electrophoresed on the same gel to serve as position makers of DNA sequences. The relative intensities of the bands on the autoradiogram of the gel were determined by the NIH Image software.

Results

Survival and mutation of plasmids

The plasmid survival, that is the capacity of the plasmid to replicate in mammalian cell, depended on the concentration of *N*-Aco-*N*-Ac-ABA (Figure 3-1). In the nucleotide excision repair deficient XP cells, the plasmid survival diminished to 2×10^{-4} at 200 μM of *N*-Aco-*N*-Ac-ABA. In contrast, the plasmid survival was 1×10^{-1} at the same concentration in DNA repair-proficient WI38-VA13. Mutation frequency increased in both cells with increasing concentration of *N*-Aco-*N*-Ac-ABA (Figure 3-2). The background plasmid mutation frequency was 2.8×10^{-4} to 4.5×10^{-4} for both cell lines, and the mutation frequency increased 50 times in normal cells and 200 times in XP cells by the treatment of the plasmid with 200 μM of *N*-Aco-*N*-Ac-ABA. Difference in the mutation frequencies between two cell lines was smaller than that in the plasmid survivals.

Base sequence analysis

The majority (85-90 %) of the *N*-Aco-*N*-Ac-ABA-induced mutations was base substitutions in both cell lines (Table 3-1). In XP cells, 61 % of the mutant plasmids had a single base substitution, while 3 % and 22 % of them had tandem (adjacent two base substitutions) and multiple (≥2 base substitutions except for the tandem base substitutions) mutations, respectively. In normal cells, the plasmids which had a single base substitution and multiple base substitutions were observed in almost the same frequency (41 % and 40 %, respectively), and three (3 %) plasmids had tandem base substitutions. Plasmids which had deletions or insertions were fewer

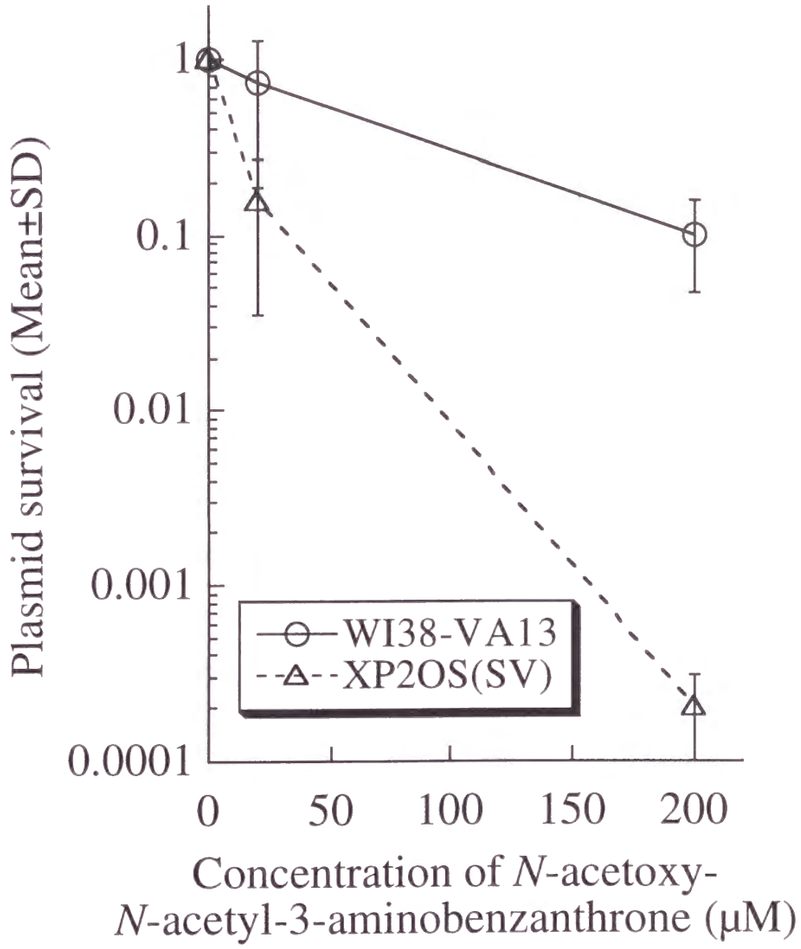


Figure 3-1. Survival of the *N*-Aco-*N*-Ac-ABA-treated pMY189 plasmids propagated in repair-proficient WI38-VA13 (○) and repair-deficient XP2OS(SV) (△). Average numbers of three independent experiments are plotted with the S.D.

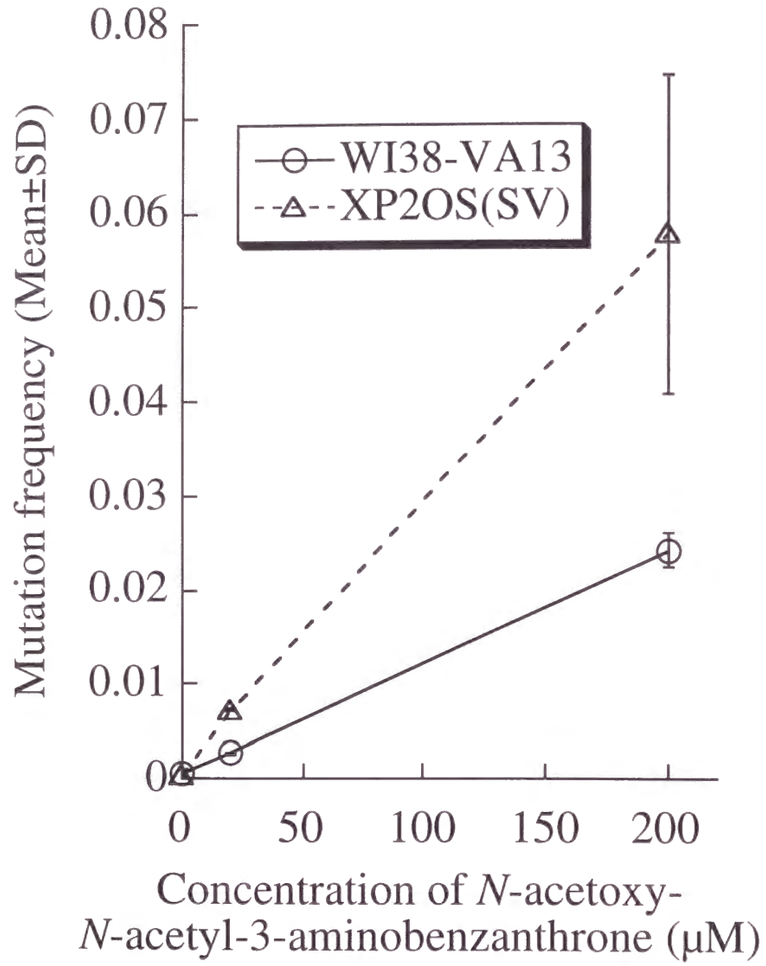


Figure 3-2. Mutation frequency of the *N*-Aco-*N*-Ac-ABA-treated pMY189 plasmids propagated in repair-proficient WI38-VA13 (○) and repair-deficient XP2OS(SV) (△). Average numbers of three independent experiments are plotted with the S.D.

Table 3-1 Types of sequence alternations in *supF* gene in *N*-Aco-*N*-Ac-ABA-treated plasmids pMY189 replicated in normal (WI38-VA13) or repair deficient (XP2OS(SV)) human fibroblasts

Types	No. of plasmids with mutations (%)	
	WI38-VA13	XP2OS(SV)
Base substitutions	99 (90)	86 (86)
Single	45 (41)	61 (61)
Tandem	10 (9)	3 (3)
Multiple*	44 (40)	22 (22)
Deletions and insertions	11 (10)	14 (14)
Single base deletion **	6 (5)	11 (11)
≥ 2 bases deletion***	5 (5)	2 (2)
Single base insertion ****	0 (0)	1 (1)
Total plasmids sequenced	110 (100)	100 (100)

Plasmids having base substitutions and deletions or insertions are listed in "Deletions and insertions".

* Two (WI38-VA13) and one (XP2OS(SV)) had accompanying tandem base substitutions.

** Five (WI38-VA13) and seven (XP2OS(SV)) had other base substitutions.

*** One (WI38-VA13) had base substitutions.

**** This plasmid (XP2OS(SV)) had a tandem base substitution.

than those with base substitutions and most of them accompanied base substitutions. (Footnotes of the table).

Types of the base substitutions are shown in Table 3-2. The majority of the base substitutions (78-83 %) occurred at guanine or cytosine in both cells. The most frequent mutations were G:C to T:A transversions and the next frequent mutations were G:C to A:T transitions in either cell line.

The author selected *supF* mutants from experiments in which mutation frequency was at least 30-fold of the control, and DNA sequence analysis of the mutants was performed. Therefore, almost all the mutations shown here do not contain spontaneous mutations.

Mutation spectrum

Locations of the *N*-Aco-*N*-Ac-ABA-induced base substitutions over the coding region of the *supF* tRNA gene are shown in Figure 3-3. The distribution was similar in normal and XP cells. Mutations were not distributed randomly ($P<0.0001$ in both cells lines) but were located mostly at specific sites. There were several hot spots (≥7 base changes in normal cells and ≥6 base changes in XP cells), at sites 109, 115, 123, 134, 144 and 159 in normal cells, and 115, 123, 127 and 159 in XP cells, where the number of mutations observed was ≥4-fold greater than the number expected for random distribution. The most prominent hot spot was at base pair 159 in both cell lines. Almost all (9 of 10) hot spots were located at the sites of G:C base pairs. Seven of 10 hot spots were at 5'-G<u>G</u>G-3' (or 5'-C<u>C</u>C-3') and 5'-<u>G</u>G-3' (or 5'-C<u>C</u>-3') sites.

Table 3-2 Types of base substitutions in *supF* gene in *N*-Aco-*N*-Ac-ABA-treated plasmids replicated in normal (WI38-VA13) or repair deficient (XP2OS(SV)) human fibroblasts

Base substitutions	No. of base changes (%)	
	WI38-VA13	XP2OS(SV)
G:C to T:A	94 (51)	58 (41)
G:C to A:T	32 (18)	34 (24)
G:C to C:G	17 (9)	24 (17)
A:T to G:C	26 (14)	17 (12)
A:T to T:A	10 (6)	7 (5)
A:T to C:G	4 (2)	1 (1)
Total	183 (100)	141 (100)

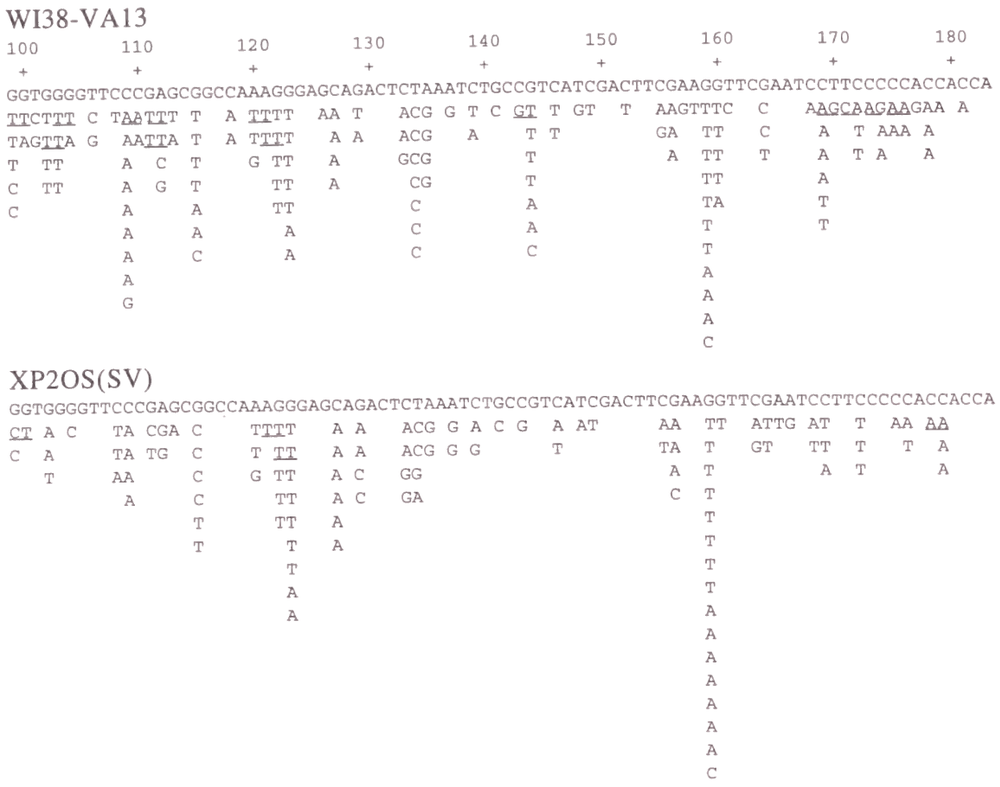


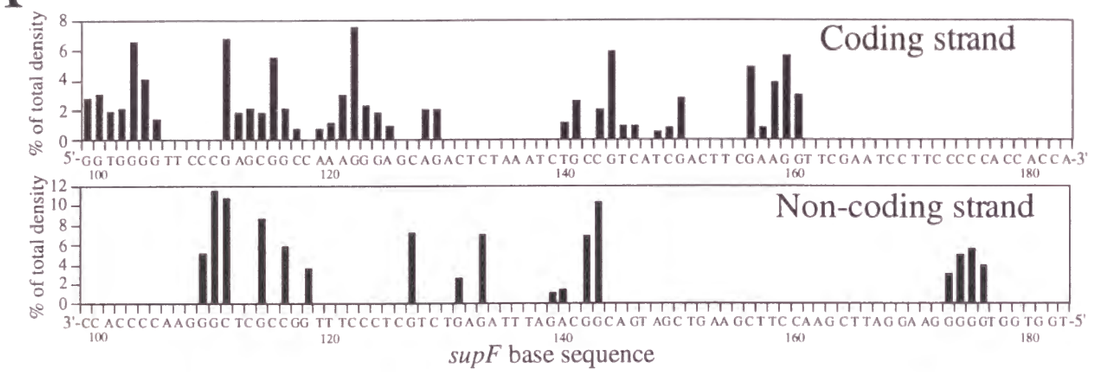
Figure 3-3. Distribution of the *N*-Aco-*N*-Ac-ABA-induced base substitutions in *supF* tRNA gene. Underlined bases are with tandem base substitutions. Sites corresponding to tRNA coding sequences (99-183) are shown. Multiple mutations, deletions and insertions are as follows (the site of the change is shown in parenthesis); WI38-VA13: T to C (17) + G to C (144); G to C (31) + C to A (109); G to T (31) + G to T (65) + G to T (72) + G to T (103) + G to T (159); C to T (35) + G to C (159); G to C (36) + C to T (75) + C to A (182); A to T (44) + G to T (115) + G to A (156); G to A (51) + G to A (100) + G to T (113); C to T (59) + C to T (139) + C to A (174); C to T (63) + G to T (160); C to A (64) + C to A (75) + C to T (169) + C to A (178); A to T (69) + C to A (85) + C to A (109); G to T (77) + G to T (150) + G to T (160); deletion (98) + G to C (99) + G to T (102); G to C (99) + G to T (102) + G to A (115); G to T (99) + G to T (102); G to T (99) + G to A (144); T to G (101) + C to A (175) + C to A (176); G to A (104) + G to C (115); T to C (106) + T to C (197); C to T (108) + G to A (126); C to A (109) + A to T (195); C to A (109) + C to A (176); C to A (109) + C to G (222); C to A (110) + C to A (118) + C to A (174); G to A (113) + G to T (122); G to A (115) + G to T (122); G to T (115) + A to T (120) + C to T (146); G to T (115) + G to T (123) + G to A (144); G to T (115) + G to T (159); C to A (118) + C to A (133); A to T (120) + A to T (121) + deletion (122); G to T (122) + C to G (149); G to T (123) + A to G (157); G to T (123) + G to C (141) + G to T (144); C to A (127) + A to G (227); G to A (129) + G to T (159); G to T (129) + G to T (160); T to C (134) + C to T (152); T to C (134) + T to C (224); A to G (135) + C to A (175); C to T (146) + C to A (169); C to G (155) + A to T (158); G to A (156) + G to T (164); deletion (168) + C to T (172); C to A (168) + C to A (179); deletion (168) + C to T (172); C to T (169) + C to A (178); T to C (171) + C to A (172) + C to A (178); deletion (172) + A to G (177) + C to A (220); deletion (96-97) + T to C (101) + G to T (103) + G to T (144) + T to C (244); deletion (150-156); deletion (91-128); deletion (59-108); deletion (33-180), XP2OS(SV): C to G (12) + C to G (79) + C to T (108) + C to A (146) + C to A (163) + C to T (176) + C to T (198); G to A (16) + C to G (133); A to G (21) + G to T (115) + A to T (121) + G to T (122); C to A (35) + C to A (127); G to C (43) + G to C (65) + G to A (102) + G to T (111); T to A (50) + G to A (51); C to A (64) + G to A (159); C to A (94) + C to A (175) + deletion (178); C to A (95) + deletion (168) + C to T (172); G to A (97) + C to T (168); G to T (102) + G to C (115); deletion (102) + C to T (108) + A to G (112) + G to A (129) + T to A (148) + G to A (156) + G to A (231); G to C (104) + G to C (129) + G to C (217); C to A (108) + T to A (134); C to A (109) + C to A (179) + T to C (224); C to A (109) + C to G (143); C to A (109) + C to A (222); G to C (111) + G to A (129); G to T (115) + G to T (123); deletion (119) + G to T (122); A to T (120) + G to T (122); G to A (123) + A to G (225); C to G (133) + C to T (149); C to T (146) + C to T (155) + C to T (169); G to T (159) + A to G (166); deletion (168) + C to T (172); deletion (168) + C to T (172); C to A (176) + C to A (179); C insertion (170-171) + C to A (178) + C to A (179); deletion (122); deletion (122); deletion (122); deletion (15-172); deletion (39-104); deletion (172);

Polymerase-stop assay

To determine if the mutation hot spots corresponded to hot spots for the mutagen-binding in the *supF* gene, the author carried out the polymerase-stop assay. The assay reveals the sites where bulky *N*-Aco-*N*-Ac-ABA adducts interfere with DNA replication. Figure 3-4A shows a diagram of the relative amounts of the binding sites as judged from intensities of the bands on the gel. The relative intensity of the bands increased as concentration of *N*-Aco-*N*-Ac-ABA was increased (Data not shown). Almost all guanines in the analyzed region of *supF* sequences had adducts, and no strong signals at other bases were detected, indicating that *N*-Aco-*N*-Ac-ABA preferentially binds to guanine residues. The hot spots of the DNA adducts were at positions 103, 111, 115, 122, 144 and 159 on the coding strand, and 110, 111, 114, and 143 on the non-coding strand.

Figure 3-4B shows a relationship between sites of DNA adduct and locations of base substitution mutation. Good correlation between the amount of adducts and the frequency of base changes was shown at positions 115, 144 and 159. However, sites 110, 111, 114 and 143 showed relatively high binding frequency, but few mutations were observed at these sites.

A



B

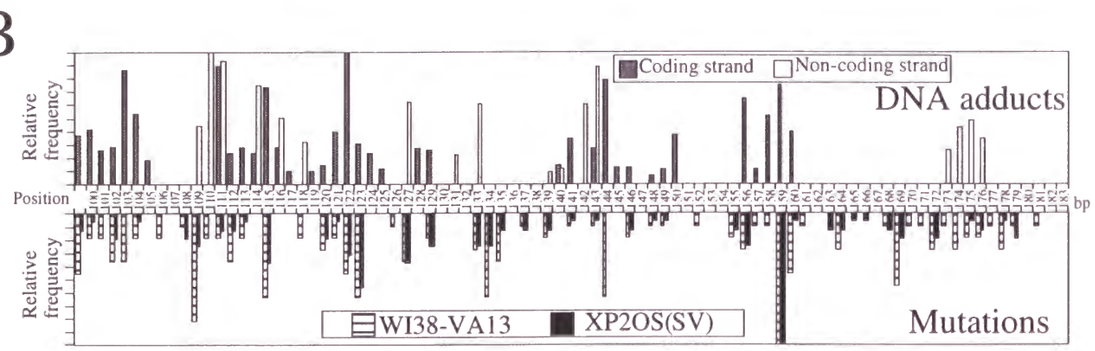


Figure 3-4. A: Relative frequency of *N*-Aco-*N*-Ac-ABA adducts, as judged by the polymerase stop assay, B: Correlation between sites of DNA adducts and locations of base substitution mutations.

Discussion

In the Ames assay, NBA is more mutagenic to the bacterial strain which have high activity of acetyltransferase than to the nitroreductase-deficient strain (1). These results indicate that the NBA is metabolized and reacts with DNA as other nitroaromatic carcinogens. In the present work, the author used synthetic *N*-Aco-*N*-Ac-ABA to yield NBA-induced DNA damage. This derivative reacts with DNA and forms the adduct dG-*N*-Ac-ABA *in vitro* (Chapter 1)(2). dG-*N*-Ac-ABA was also found in HepG2 cells treated with NBA (Chapter 2). Therefore, the author thinks that the *N*-Aco-*N*-Ac-ABA-induced DNA damage *in vitro* reflects damage induced by NBA *in vivo*, although the metabolism of NBA has not been clearly understood.

As shown in Figures 3-1 and 3-2, plasmid survival was lower and mutation frequency was higher in the repair-deficient XP cells than in normal cells. The similar results have been reported with UV (8), *cis*-diamminedichloroplatinum(II) (18) and Aflatoxin B_1 (19). These results suggest that the reduced replication efficiency and the enhanced mutation frequency by *N*-Aco-*N*-Ac-ABA are due to unrepaired DNA adducts in the cells, and *N*-Aco-*N*-Ac-ABA adducts are removed from DNA by the nucleotide excision repair.

In *Salmonella typhimurium*, NBA is a strong inducer of frame shift mutations (1). In contrast, majority of the mutations observed in the plasmids propagated in human cells was base substitutions (Table 3-1). Similar difference in the mutational specificity is shown with acetylaminofluorene (AAF), 1,8-DNP and 1,6-dinitropyrene (1,6-DNP)(17)(20)(21)(22). The inconsistency could result from a difference in the

target gene sequence or a difference in mechanisms of mutagenesis between bacterial and mammalian cells (17)(20). However, in present the author cannot eliminate a possibility that DNA lesions produced by NBA may be different from those by *N*-Aco-*N*-Ac-ABA.

In the *N*-Aco-*N*-Ac-ABA-induced mutant plasmids, 40 % and 22 % had multiple mutations in normal and XP cells, respectively (Table 3-1). To yield mutant plasmids with enough higher mutation frequency than the background mutation frequency, the plasmids were reacted with *N*-Aco-*N*-Ac-ABA at higher concentration for normal cell lines (0.2-2 mM) than for XP cell lines (20 - 200 μM). Such severe treatments for normal cells may have formed abundant multiple lesions yielding the multiple mutations.

The majority of the base substitutions (78-83 %) induced by *N*-Aco-*N*-Ac-ABA occurred at G:C sites and the most prevalent base change (41-51%) was the G:C to T:A transversion in both cell lines. These results are similar to those by other nitroaromatic carcinogens that have been studied by the shuttle vector system. The fraction of G:C to T:A was 65 % by aminofluorene (AF) (20), 65 % by AAF (20), 61 % by 1-nitropyrene (1-NP) (16), 64 % by 1,6-DNP (17) and 61 % by 1-nitrosopyrene (1-NOP) (23). In this study, the base changes occurred preferentially at certain sites where the sequence is 5'-G<u>G</u>G-3' or 5'-C<u>C</u>C-3' (positions 109, 123, and 159), and 5'-<u>G</u>G-3' or 5'-C<u>C</u>-3' (position 115) (Figure 3-3). The position 109 is also a hot spot by AF, 1-NP, 1,6-DNP and 1-NOP, and the positions 123 and 159 are hot spots by 1-NP, 1,6-DNP and 1-NOP (16)(17)(20)(23). The result of polymerase-stop assay indicated that *N*-Aco-*N*-Ac-ABA preferentially bound to guanine residues as

other nitroaromatic compounds and suggested that the spectra of mutations reflect, at least in part, the preferential binding of *N*-Aco-*N*-Ac-ABA to specific sites (positions 115, 144 and 159). However, few or no mutations were induced at some of the sites where adducts were frequently formed. This suggests that other factors such as DNA repair and the neighboring base sequences may affect the spectra of mutagenesis in addition to the preferential binding to guanine residues.

The carcinogenicity of NBA has not been demonstrated. However, it is suspected to be carcinogenic to humans because micronucleus formation in mouse as well as mutagenicity to bacteria were reported (1). Specific mutations have been shown to be critical for the activation of oncogenes and inactivation of tumor suppressor genes in human cancers. The majority of human cancers have point mutations in the *p53* tumor suppressor gene (24), and the specificity in the types of the mutations in this gene is related to causal environmental agents, *e.g.*, the C to T transitions in skin tumors by sunlight UV (25)(26), and the G to T transversions in liver tumors by aflatoxinB_1 (19)(27). There is no previous report to describe mutagenic specificity of NBA. If environmental NBA is a human carcinogen, the mutation pattern shown here will help to predict and identify NBA-made signatures in genes associated with NBA-induced human carcinogenesis.

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CHAPTER 4

A spectrum of mutations induced by crotonaldehyde in shuttle vector plasmids propagated in human cells

Abstract

A spectrum of crotonaldehyde-induced mutations in the *supF* gene of the shuttle vector plasmid pMY189 replicated in human fibroblast cells was examined. Base sequence analysis of 104 plasmids with mutations in the *supF* gene revealed that the majority of the mutations were base substitutions (85%) and the rest were frameshifts (15%). A single base substitution was most frequently found (47%), while 25% had multiple base substitutions and interestingly 13% had tandem (adjacent two) base substitutions. Of the base substitution mutations, 50% were G:C to T:A transversions and 23% were G:C to A:T transitions. The mutations were not distributed randomly but were located at several hotspots, most of which were G:C base pair in 5'-AAGG-3' (or 5'-CCTT-3') sequences. Production of propanodeoxyguanosine adducts may be related to such specificity in the mutation spectrum.

Introduction

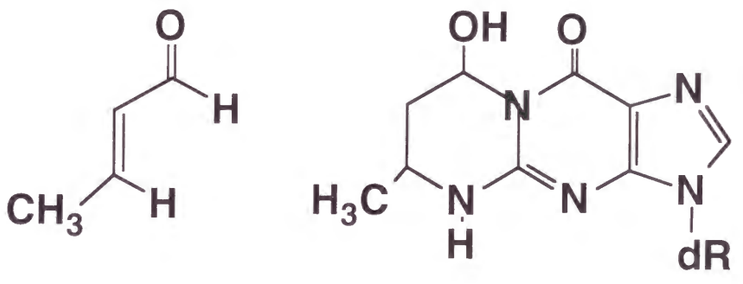
Crotonaldehyde (Figure 4-1) is one of the α,β-unsaturated carbonyl compounds which are present in our environments as commonly-used industrial chemicals, natural products of widespread occurrence, environmental contaminants and products of the endogenous metabolism in human beings (1). Crotonaldehyde is mutagenic to bacteria *Salmonella typhimurium* without metabolic activation (2) and carcinogenic to rats (3).

Crotonaldehyde reacts with DNA bases *in vitro*, producing 8-hydroxy-6-methyl-1,N^2-propanodeoxyguanosine, a cyclic 1,N^2 adduct (4). This crotonaldehyde-derived 1,N^2-propanodeoxyguanosine adduct (CRdG) (Figure 4-1) is detected in animals exposed to carcinogens such as cyclophosphamide and *N*-nitrosopyrrolidine (NPYR) (5,6). Foiles *et al*. showed that CRdG was present in DNA of cultured Chinese hamster ovary cells treated with crotonaldehyde (7). CRdG was also formed in DNA of various tissues from humans and rodents without any specific treatment, presumably due to the endogenous formation (8,9).

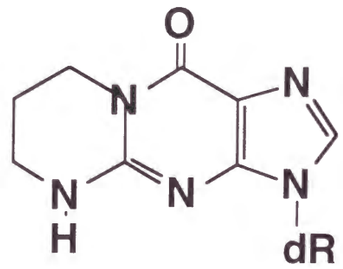
Site-specific mutagenesis studies using 1,N^2-propanodeoxyguanosine (PdG)(Figure 4-1), which has a structure similar to CRdG, demonstrated that the propano adduct caused frameshifts (deletions) and base substitutions in DNA propagated in bacteria and mammalian cells. When viral DNA containing PdG in the CG repeats was introduced into *Escherichia coli*, frameshift mutations were frequently detected (10). When plasmids containing the PdG were replicated in simian kidney (COS) cells or in *E. coli*, the predominant base substitution was PdG to T in both cells, and significantly high frequency of PdG to A substitution was also

reported in *E. coli* (11-13).

The author intends to produce CRdG in DNA by treating the plasmids with crotonaldehyde. The CRdG may cause mutations as PdG, with specificity as mentioned above. Mutation spectra caused in the *supF* gene in the plasmids pMY189 (14) were investigated.



Crotonaldehyde **CRdG**



PdG

Figure 4-1. Structures of Crotonaldehyde, CRdG and PdG.

Materials and methods

Chemicals

Crotonaldehyde, ampicillin, chloramphenicol, nalidixic acid, isopropyl-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) were obtained from Wako Chemicals (Osaka, Japan). The company guarantees that purity of crotonaldehyde is higher than 99%. LIPOFECTAMINE™ Reagent was purchased from GIBCO BRL (Gaithersburg, MD). Restriction endonuclease *Dpn*I was obtained from New England Biolabs, Inc (Beverly, MA). QIAGEN plasmid-kit and QIAprep-spin Plasmid kit were purchased from QIAGEN GmbH (Hilden, Germany).

Cells

SV40-transformed normal human fibroblast cell line WI38-VA13 (15) was obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in Dulbecco's modified minimum essential medium (Nikken, Kyoto, Japan) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT).

Shuttle vector plasmid and Bacterial strains

The shuttle vector plasmid pMY189, previously constructed by Matsuda *et al.* (14), was used for analysis of mutations induced by crotonaldehyde. The pMY189 was derived from the pZ189 (16) as described previously (14). The indicator *E.coli* strain KS40/pKY241 (17) was kindly supplied by Dr. S. Akasaka, Division of Industrial Health, Osaka Prefectural Institute of Public Health, Osaka, Japan. KS40

is a nalidixic acid-resistant (*gyrA*) derivative of MBM7070 (*lacZ* (am) *CA7070 lacY1 HsdR HsdM* Δ*(araABC-leu)7679 galU galK rpsL thi*)(18), which has been used for detection of the mutated pZ189. The plasmid pKY241 was constructed by Akasaka *et al.* (17) and contains a chloramphenicol resistant marker and a *gyrA* (amber) gene. *E.coli* KS40/pKY241 cells carrying the active *supF* gene are sensitive to nalidixic acid, whereas the cells carrying the mutated *supF* form colonies on plates containing nalidixic acid, chloramphenicol and ampicillin. *E.coli* cells containing the active *supF* gene produce blue colonies, whereas cells having the mutated *supF* gene produce white or light blue colonies on the selection plates.

Treatment of plasmids with crotonaldehyde, transfection to human cells, and plasmid recovery

Purified stocks of pMY189 were prepared by using the QIAGEN plasmid purification kit. The plasmids (12 μg) were treated with various concentrations of crotonaldehyde in total volume of 0.5 mL of 0.1 M sodium phosphate buffer (pH7.4). The reaction was allowed to proceed for five days at 37℃. At the end of the reaction, pH of the reaction buffer was still 7.4. The plasmids were precipitated with ethanol to remove the non-reacted excess crotonaldehyde, and were redissolved in 60μL of water.

The human cells WI38-VA13 (80% confluent in a 60mm tissue culture plate) were transfected with 4 μg of the crotonaldehyde-treated pMY189 DNA, with LIPOFECTAMINE™ Reagent. The cells were incubated at 37°C for 72 hours in a CO_2 incubator. Then plasmids were extracted from the cells using QIAprep-spin

Plasmid kit. The purified plasmids were digested with the restriction endonuclease *Dpn*I to eliminate the non-replicated plasmids which retain the bacterial methylation pattern.

Selection of mutated supF, and determination of DNA base sequences

Plasmid DNA was introduced into the indicator bacteria KS40/pKY241 by the electroporation apparatus *E.coli* Pulser (Bio-Rad Laboratories, Hercules, CA). The bacteria cells were plated on LB agar containing nalidixic acid, ampicillin and chloramphenicol at concentrations of 50 μg/mL, 150 μg/mL and 30 μg/mL, respectively, supplemented with IPTG and X-gal to select the plasmids containing the mutated *supF* genes. A portion of the cells was plated on LB agar containing ampicillin and chloramphenicol to measure the total number of transformants. After incubation for 24 hours at 37°C colonies were counted and mutation frequencies were calculated.

Mutated plasmids were extracted and purified from the overnight culture with the QIAprep-spin Plasmid kit and the size of each mutated plasmid was checked by agarose gel electrophoresis. The base sequences of the *supF* gene of the plasmids which were not aberrant in size were determined with the ABI PRISM™ Dye Primer (-21M13) Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Corporation, Foster, CA) using the 370A automatic DNA sequencer (Perkin-Elmer Corporation, Foster, CA).

The χ^2-goodness of fit test was used to determine if crotonaldehyde-induced base substitution mutations were distributed randomly or non-randomly over the

coding region of the *supF* tRNA gene.

Results

Survival and mutation of plasmids

Crotonaldehyde-treated pMY189 plasmids transfected into the human fibroblast cells were incubated for three days to allow repair, mutation fixation, and replication. Then the plasmids were extracted, and the indicator *E.coli* cells were transformed by the plasmids to detect survival and the crotonaldehyde-induced mutation (Figure 4-2). The crotonaldehyde-treatment of pMY189 caused reduction in the ampicillin-resistant colonies, and enhanced mutation frequency of the *supF* gene. The background mutation frequency of the *supF* gene was 1.1×10^{-3}.

Base sequence analysis

Base sequences of the *supF* marker gene of the 104 plasmids of normal size were determined. Majority (85%) of the mutations were base substitutions, whereas only 15% showed frameshift mutations (Table 4-1). About half of the mutant plasmids had a single base substitution, while 13% had tandem (adjacent two base substitutions) and 25% had multiple (≥2 base substitutions except for the tandem base substitutions) mutations. Sixteen (15%) plasmids had frameshift mutations and six of them had base substitutions as well.

Types of the base substitutions are shown in Table 4-2. Majority of the base substitutions (86%) were with guanine or cytosine. The most frequent mutations were G:C to T:A transversions. The next predominant mutations were G:C to A:T transitions, followed by G:C to C:G transversions.

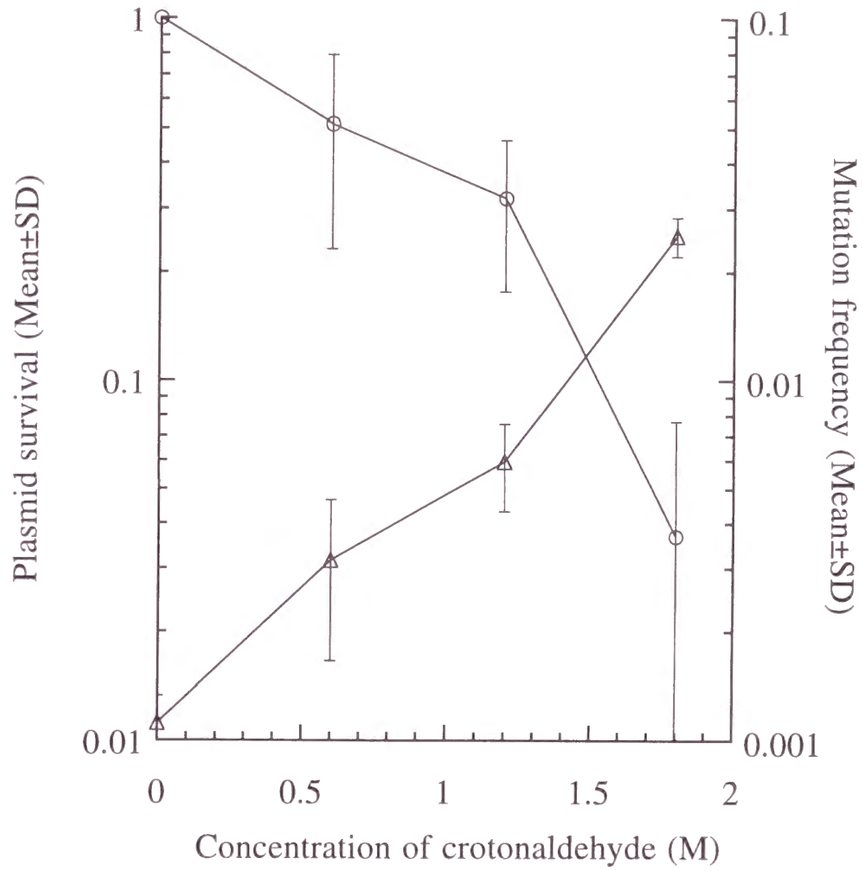


Figure 4-2. Plasmid survival (○) and mutation (△) of the crotonaldehyde-treated plasmids pMY189 propagated in normal human fibroblasts. Average numbers of three independent experiments are plotted with the S.D. of the mean.

Table 4-1 Types of sequence alternations in *supF* gene in crotonaldehyde-treated plasmids pMY189 replicated in normal human fibroblasts

Types	No. of plasmids with mutations (%)
Base substitutions	88 (85)
Single	49 (47)
Tandem	12 (12)
Multiple*	27 (26)
Frameshifts	16 (15)
Single base deletion **	5 (5)
≥ 2 bases deletion***	9 (9)
Single base insertion ****	1 (1)
≥ 2 bases insertion	1 (1)
Total plasmids sequenced	104 (100)

Plasmids with frameshifts are listed in "Frameshifts" only.

* Three had accompanying tandem base substitutions.

** Four had other base substitutions.

*** One had a base substitution.

**** This plasmid had base substitutions.

Table 4-2 Types of base substitutions in *supF* gene in crotonaldehyde-treated plasmids replicated in normal human fibroblasts

Base substitutions	No. of base changes (%)
G:C to T:A	70 (50)
G:C to A:T	32 (23)
G:C to C:G	19 (13)
A:T to G:C	13 (9)
A:T to T:A	5 (4)
A:T to C:G	3 (2)
Total	142 (100)

Mutation spectrum

Distribution of the crotonaldehyde-induced base substitutions over the coding region of the *supF* tRNA gene is shown in Figure 4-3. Identical mutants derived from the same transfection plate were not scored to exclude clusters. Mutations were not distributed randomly ($P<0.0005$) but were located mostly at certain specific sites. There were four hotspots (>5 mutations), at sites 123, 133, 168 and 169, where the number of mutations observed was 4-fold or more greater than the number expected for random distribution. The most prominent hotspot was at base pair 133, at which almost all base substitutions (13 of 14) were transversions. All hotspots were located at the sites of G:C base pairs. Three of four hotspots (123, 168 and 169) were at the sites of G:C base pair in 5'-AAGG-3' (or 5'-CCTT-3') sequences.

There were 16 tandem base substitutions. Five were GG to TT (or CC to AA) base substitutions. Two GG to AA (or CC to TT) and two GG to AT (or CC to AT) base substitutions were next predominant tandem base substitutions. Other tandem base substitutions were a GG to CT (CC to AG), a GG to TA (CC to TA), a GG to TC (CC to GA), a GT to AA (AC to TT), a GT to TA (AC to TA), a GA to AC (TC to GT), and a AG to TT (CT to AA). Most of the tandem base substitutions (12 of 16) occurred at GG (or CC) sites.

The multiple mutations and frameshift mutations were listed in the legend of Figure 4-3. Among twenty-one base substitutions at A:T sites (Table 4-1), sixteen were found in the same gene which have multiple mutations or frameshift mutations.

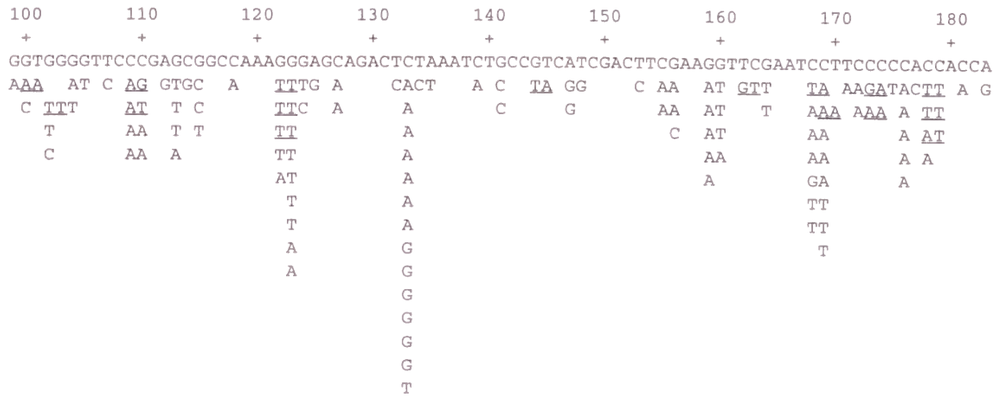


Figure 4-3. Distribution of the crotonaldehyde-induced base substitutions in *supF* tRNA gene. Underlined are tandem base substitutions. Sites corresponding to tRNA (99-183) are shown. Multiple mutations and frameshift mutations found are as follows (the site of the change is shown in parenthesis); C to T (12) & C to A (109) & C to T (110) ; C to T (38) & G to T (123) ; G to C (43) & G to A (159) ; C to T (59) & C to A (133) ; G to A (99) & G to T (102) ; G to C (100) & C to A (173) & C to A (174) ; G to C (102) & G to T (105) ; G to T (104) & T to C (132) ; T to C (107) & G to C (115) ; C to A (110) & G to A (113) ; C to A (110) & C to A (178) ; G to T (115) & G to T (122) ; C to A (118) & C to A (133) ; G to A (123) & A to G (125) ; C to T (133) & A to T (135) ; C to G (133) & A to G (147) ; C to G (133) & T to G (148) ; A to G (147) & C to A (172) ; T to C (153) & C to A (176) ; C to A (155) & C to T (175) ; G to A (159) & A to G (210) ; C to T (168) &T to C (190) ; C to A (169) & T to A (170) & C to T (172) & A to C (177) & C to A (181); C to T (169) & C to A (176) ; C to T (169) & C to A (176) ; C to T (169) & C to A (176) ; C to A (176) & C to T (178) & C to T (179); deletion (1-230) ; deletion (17-166) ; T to C (23) & T to C (24) & deletion (102) ; deletion (31-122) ; deletion (33-44) & C to A (109) ; G to T (70) & GGG to CA (122-124) ; deletion (84-121) ; deletion (93-151) ; CC to A (108-109) & A to G (112) & C to G (114) ; GG to T (102-103) & G to A (122) ; deletion (113-237 or 114-238) ; deletion (118-121) ; deletion (131-141 or 132-142) ; deletion (141-147) & A to G (183) ; large insertion (156-) ; G to T (160) & T insertion (183-184) & C to A (212) ; deletion (172)

Discussion

Eder and Hoffman reported that crotonaldehyde forms adducts to deoxyguanosine residues by the reaction to N7 and C8 as well as to N1 and N^2 atoms (19). They also detected bis-crotonaldehyde-deoxyguanosine adducts formed by the reaction to N1,N^2 and N7,C8 atoms (19). In the present study, the majority (86%) of the crotonaldehyde-induced base substitutions took place at guanine or cytosine residues, being consistent with previous studies (Table 4-2). Previous studies using PdG, which has a similar structure to CRdG, showed that the propano adducts primarily cause G to T transversions in both mammalian cells and *E.coli*, and secondarily G to A transitions in *E. coli* (11-13). As given in Table II, the most frequently identified base substitutions were G:C to T:A transversions and the next predominant mutation were G:C to A:T transitions. Such specificity in the base substitutions caused by crotonaldehyde is similar to that of PdG, possibly due to the structural similarity between PdG and CRdG. Benamira *et al.* reported that when the viral DNA containing a PdG in a CG repeat was introduced into *E. coli*, frameshift mutations (a two base pair deletion in the CG repeat) were frequently detected and these mutations could be explained by the slipped-mispairing model proposed by Streissinger (10). Such two base pair deletions were not detected in the CRdG induced mutant plasmids (Figure 4-3, *legend*), presumably because CG repeat sequences are not present in the coding region of the *supF* tRNA gene.

Sixteen of the 104 mutant plasmids (15%) contained tandem base substitutions and most of these tandem base substitutions (12 of 16) occurred at GG (or CC) sites. There is no previous report that crotonaldehyde or propano

adduct induces tandem base substitutions.

Multiple mutations were found in one forth of the mutant plasmids sequenced. In our study, the plasmids were reacted with crotonaldehyde at high concentration (0.6-1.8M) for long time (five days) to detect crotonaldehyde-induced mutation, because short reaction time (up to 24 hours) yielded mutant plasmids with just two or three times higher mutation frequency than that of background. Such a severe treatment may have formed multiple lesions and induced multiple mutations. Multiple mutations were also suggested to be linked to the activity of the error-prone DNA polymerase (20). Although no previous report that crotonaldehyde react with deoxyadenosine or deoxythymine, 21 base substitutions at A:T sites were found (Table 4-2). However, nineteen of them had accompanying other base substitutions at G:C sites or base deletions. These base changes at A:T sites might be associated with the untargeted mutations caused by the polymerase. On the other hand, Marnett *et al.* reported that crotonaldehyde induced base pair substitution mutation without the metabolic activation in *Salmonella* TA104 in which the mutational site is the nonsense sequence TAA (21). Acrolein, which has a similar molecular formula to crotonaldehyde, produces 1,N^6-propanpdeoxyadenosine as well as guanosine adducts(22). Crotonaldehyde may react with deoxyadenosine as well as deoxyguanosine and may lead to mutation at A:T sites.

There were four hotspots for base substitutions induced by crotonaldehyde. The most prominent site was at base pair 133. Other three hotspots were located in the 5'-AAGG-3' (or 5'-CCTT-3') sequences, which are same as the hotspots by 2-chloroacetaldehyde, an ultimate carcinogenic form of vinyl chloride (14). The

mechanisms responsible for the specificity at the 4 base sequences are not known. DNA damage may be induced or repair may be blocked selectively on these sequences by unknown mechanisms.

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CHAPTER 5

Spectrum of mutations in shuttle vectors and DNA intra-strand crosslinks induced by acrolein

Abstract

Types of mutations induced by acrolein in the *supF* gene on the shuttle vector plasmid pMY189 replicated in normal human fibroblast cells were examined. Base sequence analysis of 92 plasmids with mutations in the *supF* gene revealed that the majority of the mutations were base substitutions (76%) and the rest were deletions and insertions (24%). Single base substitutions were most frequently found (46%), while multiple base substitutions were 18% and tandem (two adjacent) base substitutions were 12% of the mutations. Of the base substitution mutations, G:C to T:A transversions were 44% and G:C to A:T transitions were 24%. The mutations were distributed not randomly but located at several hotspots. Acrolein produced DNA intra-strand crosslinks between guanine residues, which might be responsible for the induction of the tandem base substitution mutations.

Introduction

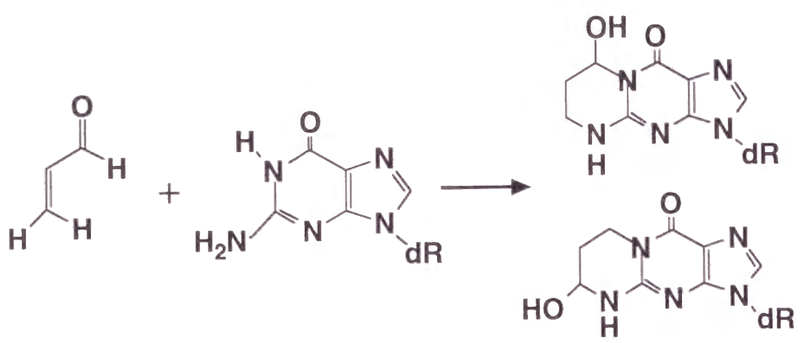
Acrolein (Figure 5-1) is one of the α,β-unsaturated carbonyl compounds which are present in our environment as commonly-used industrial chemicals, natural products of widespread occurrence, environmental contaminants and products of the endogenous metabolism in human beings (1). Acrolein is a strong DNA damaging agent in *Bacillus subtilis* Rec-assay(2), and is mutagenic to bacteria *Salmonella typhimurium* (3) and to fibroblast cells cultured from xeroderma pigmentosum (XP) patients (4) without metabolic activation.

Reaction of acrolein with deoxyguanosine has been shown to result in formation of 6-hydroxy 1,N^2-propanodeoxyguanosine and 8-hydroxy 1,N^2-propanodeoxyguanosine adducts (Figure 5-1) *in vitro* (5). These acrolein-derived hydroxypropanodeoxyguanosines (AdGs) are detected in DNA of cultured human fibroblast cells treated with acrolein and in peripheral blood lymphocytes obtained from a dog given cyclophosphamide (6). AdGs are present in DNA of various human and rodent tissues without any carcinogen treatment (7) as well as in DNA of cultured Chinese hamster ovary cells treated with acrolein (8,9). Metabolic conversion yielding AdGs was suggested in these studies.

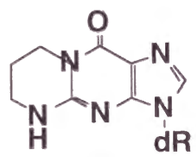
Site-directed mutagenesis studies with viral or plasmid DNA using 1,N^2-propanodeoxyguanosine (PdG)(Figure 5-1), which has a structure similar to AdGs, demonstrate that the PdG causes frameshifts (deletions) and base substitutions in the DNA propagated in bacteria and mammalian cells. When viral DNA containing PdG in the CG repeats is introduced into *Escherichia coli*, frameshift mutations are frequently detected (10). When plasmids containing the

PdG are replicated in simian kidney (COS) cells or in *E. coli*, the predominant base substitution is PdG to T in both cells, and significantly high frequency of PdG to A substitution in the plasmids is also reported in *E. coli* (11-13).

Although the mutational specificity of PdG is well known, the spectrum of mutations by acrolein is still unknown. The author intends to produce DNA damages in shuttle vector plasmids pMY189 by treating the plasmids with acrolein , and frequencies and types of mutations in the *supF* gene of the plasmids in human cells induced by acrolein were investigated.



Acrolein deoxyguanosine AdGs



PdG

Figure 5-1. Acrolein, two forms of acrolein-derived hydroxy 1, N^2-propanodeoxygua nosine (AdGs) and propanodeoxyguanosine (PdG).

Materials and methods

Chemicals

Acrolein (purity > 99.9 %), ampicillin, chloramphenicol, nalidixic acid, isopropyl-β-D-thiogalactoside (IPTG) and 5-bromō-4-chloro-3-indoyl-β-D-galactoside (X-gal) were obtained from Wako Chemicals (Osaka, Japan). Restriction endonuclease *Dpn*I was purchased from New England Biolabs, Inc (Beverly, MA). QIAGEN plasmid-kit, QIAprep-spin Plasmid kit and QIAquick-spin PCR Purification kit were obtained from QIAGEN GmbH (Hilden, Germany). Three kinds of 20 mer single-strand DNA (*i.e.*, 5'-TCGTGACTGGGAAAACCCTG-3', 5'-GCGTTACCCAACTTAATCGC-3', 5'-CTTGCAGCACATCCCCCTTT-3') and 5'-Biotinated 20 mer single-strand DNA (*i.e.*, 5'-TTAACGCGAATTTTAACAAA-3') were synthesized by Nippon Seihun Corp. (Tokyo, Japan). pBluescript KS(-) was purchased from Toyobo Corp. (Tokyo, Japan). Taq polymerase was obtained from Takara Shuzo Corp. (Kyoto, Japan). Dynabeads M-280 streptavidin was obtained from DYNAL A.S. (Oslo, Norway). [γ-^{32}P] ATP was purchased from Amersham Corp. (Chiba, Japan).

Cells

An SV40-transformed normal human fibroblast cell line WI38-VA13 (14) was obtained from the American Type Culture Collection (Rockville, MD). DNA repair deficient XP2OS(SV) cells were established from a Japanese group A XP patient (15). All cells were cultured in Dulbecco's modified minimum essential medium (Nikken, Kyoto, Japan) supplemented with 10% fetal bovine serum (Hyclone, Logan,

UT).

Shuttle vector plasmid and Bacterial strains

The shuttle vector plasmid pMY189, constructed by Matsuda *et al.* (16), was used for analysis of mutations. The pMY189 was derived from the pZ189 (17) as described previously (16). The indicator *E.coli* strain KS40/pKY241 (18) is a nalidixic acid-resistant (*gyrA*) derivative of MBM7070 (*lacZ* (am) *CA7070 lacY1 HsdR HsdM* Δ*(araABC-leu)7679 galU galK rpsL thi*)(19), which has been used for detection of the mutated pZ189. The plasmid pKY241 was constructed by Akasaka *et al.* (18) and contains a chloramphenicol resistant marker and a *gyrA* (amber) gene. *E.coli* KS40/pKY241 cells carrying the active *supF* gene are sensitive to nalidixic acid, whereas the cells carrying the mutated *supF* form colonies on the selection plates containing nalidixic acid, chloramphenicol and ampicillin. *E.coli* cells containing the active *supF* gene produce blue colonies, whereas cells having the mutated *supF* gene produce white or light blue colonies on the selection plates.

Treatment of plasmids with acrolein, transfection to human cells, and plasmid recovery

Purified stocks of pMY189 were prepared by using the QIAGEN plasmid purification kit. The plasmids (30 μg) were treated with various concentrations of acrolein in total volume of 0.5 mL of 0.1M sodium phosphate buffer (pH7.4). The reaction was allowed to proceed for 15 hours at 37°C followed by ethanol precipitation of the plasmids to remove the non-reacted excess acrolein, and the

plasmids were redissolved in 400 μL of Dulbecco's phosphate buffered saline (PBS) solution (pH 7.5).

The human cells ($2x10^7$), WI38-VA13 or XP2OS(SV), and 15 μg acrolein-treated pMY189 in PBS solution (200 μL) were placed in an electroporation chamber (electrodes 0.3 cm apart) (PDS, Inc., Madison, WI) and the cells were transfected with the plasmids by electric pulses (600 V, 4 times). The cells were plated in 10-cm dishes and incubated at 37°C for 72 hours in a CO_2 incubator. Then plasmids were extracted from the cells using the QIAprep-spin Plasmid kit. The purified plasmids were digested with the restriction endonuclease *Dpn*I to eliminate non-replicated plasmids which retain the bacterial methylation pattern.

Selection of mutated supF, and determination of DNA base sequences

Plasmid DNA replicated in the human cells was introduced into the indicator bacteria KS40/pKY241 by the electroporation apparatus *E.coli* Pulsar (Bio-Rad Laboratories, Hercules, CA). The bacteria cells were plated on LB agar containing 50 μg/mL nalidixic acid, 150 μg/mL ampicillin and 30 μg/mL chloramphenicol, IPTG and X-gal to select the plasmids containing the mutated *supF* genes. A portion of the cells was plated on LB agar containing ampicillin and chloramphenicol to measure the total number of transformants. After the plates were incubated for 24 hours at 37°C, colonies were counted and mutation frequencies were calculated.

E. coli having mutated plasmids which had been propagated in normal cells were cultured overnight. Then the plasmids were extracted and purified with the QIAprep-spin Plasmid kit and the size of the mutated plasmid was checked by

agarose gel electrophoresis. The base sequences of the *supF* gene of the plasmids which were not aberrant in size were determined with the ABI PRISM™ Dye Primer (-21M13) Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Corporation, Foster, CA) using the 370A automatic DNA sequencer (Perkin-Elmer Corporation, Foster, CA). The mutants having the identical base changes derived from the same transfection plate were not scored to exclude clusters of the same clones.

The χ^2-goodness of fit test was used to determine if the acrolein-induced base substitution mutations were distributed randomly or non-randomly over the coding region of the *supF* tRNA gene.

Detection of intra-strand crosslinks

A part of pBluescript KS(-) sequences (175 base pairs) was amplified with TaKaRa Ex Taq polymerase (total reaction volume was 50μL) by the polymerase chain reaction (PCR) using following two primers: 5'-TCGTGACTGGGAAAACCCTG-3' and 5'- Biotinated-TTAACGCGAATTTT AACAAA-3'. The PCR conditions were composed of 30 cycles of 94°C for 0.5 min, 55°C for 0.5 min and 72°C for 1 min. The PCR products were purified with the QIAquick-spin PCR Purification kit. About 14 pmol of the PCR products were obtained. These biotinated 175 mer double stranded-DNA were immobilized to Dynabeads M-280 streptavidin. After the 175 mer DNA was denatured with 0.1M NaOH and washed with 0.1 M NaOH and TE buffer containing 1M NaCl on a magnet, single stranded 175 mer DNA immobilized to Dynabeads M-280

streptavidin were obtained. The immobilized DNA sequences are as follows:

5'-immobilized-TTAACGCGAATTTTAACAAAATATTAACGCTTACAATTTCC ATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGG CCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCA AGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGA-3'

Two kinds of DNA (*i.e.*, 5'-TCGTGACTGGGAAAACCCTG-3' and 5'-GCGTTACCCAACTTAATCGC-3') were labeled with ^{32}P by treatment with bacteriophage T4 polynucleotide kinase and [γ-^{32}P] ATP. The immobilized single-stranded 175 mer DNA was annealed with ^{32}P labeled 5'-TCGTGACTGGGAAAACCCTG-3' and non-labeled 5'-GCGTTACCCAACTTAA TCGC-3', or with ^{32}P labeled 5'-GCGTTACCCAACTTAATCGC-3' and non-labeled 5'-CTTGCAGCACATCCCCCTTT-3', by heating and cooling in 1M NaCl for detecting a GG-intrastrand crosslink or a CC-intrastrand crosslink, respectively (Figure 5-5, "1 Preparation of substrates").

The annealed DNA (1 pmol of each) was treated with 54 mM of acrolein in 0.05 M sodium phosphate buffer (pH7.4) containing 0.1M NaCl for 24 hours at 4°C (Figure 5-5, "2 Treatment of substrates with acrolein"). DNA was collected by a magnet and redissolved in 10 μL of 0.1M NaCl. Then DNA solution was mixed with 2μL of dye (50% urea, 15% glycerol, 0.25% buromophenolblue, 0.25% xylenecyanol), and separated by 12% polyacrylamide gel (50% urea, 1xTBE) electrophoresis for 45 minutes at 500V. The gel was exposed to X-ray films for autoradiography (Figure 5-5, "3 PAGE & Autoradiography").

Results

Survival and mutation of plasmids

The acrolein-treated plasmids were inactivated with increasing concentration of acrolein. They were apparently not subjected to the nucleotide excision repair as there were no difference in the plasmid survival when they were propagated in normal and XP cells (Figure 5-2). Lack of the repair was also demonstrated in the acrolein induced mutations when the mutations were detected after the acrolein-treated plasmids were propagated in normal and XP cells (Figure 5-3).

Base sequence analysis

Types of mutation in the *supF* gene on the plasmids replicated in normal cells were examined. The majority (76%) of the mutations were base substitutions (Table 5-1). About half of the mutant plasmids had a single base substitution, while 12% and 18% of them had tandem (adjacent two base substitutions) and multiple (≥2 base substitutions except for the tandem base substitutions) mutations, respectively. Twenty two (24%) plasmids had deletions or insertions and five of them had base substitutions as well.

Types of the base substitutions are shown in Table 5-2. The majority of the base substitutions (80%) occurred at guanine or cytosine. The most frequent mutations were G:C to T:A transversions (44%), and the next frequent mutations were G:C to A:T transitions (24%), followed by G:C to C:G and A:T to T:A transversions (12% each).

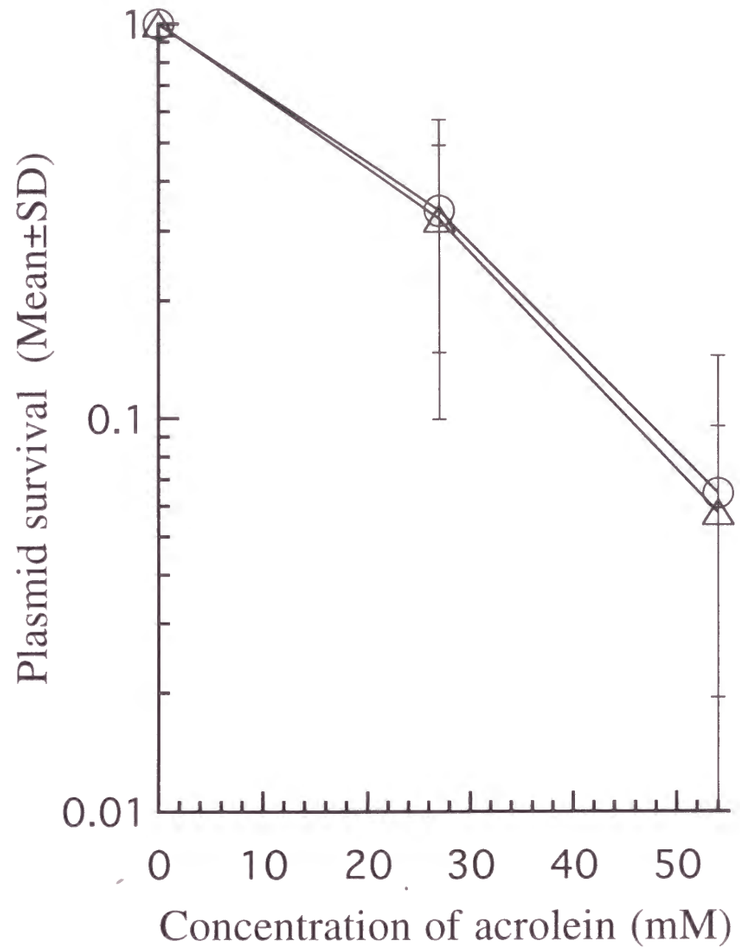


Figure 5-2. Survival of the acrolein-treated pMY189 plasmids propagated in repair-proficient WI38-VA13 (○) and repair-deficient XP2OS(SV) (△). Average numbers of three independent experiments are plotted with the S.D.

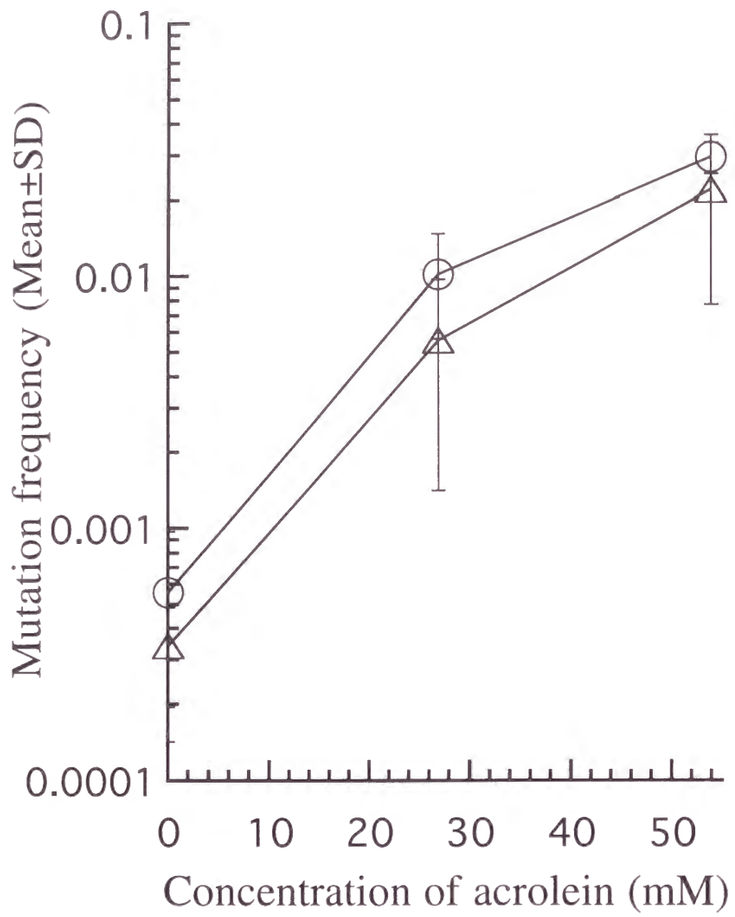


Figure 5-3. Mutation frequency of the acrolein-treated pMY189 plasmids propagated in repair-proficient WI38-VA13 (○) and repair-deficient XP2OS(SV) (△). Average numbers of three independent experiments are plotted with the S.D.

Table 5-1 Types of sequence alternations in *supF* gene in acrolein-treated plasmids pMY189 replicated in normal human fibroblasts

Types	No. of plasmids with mutations (%)
Base substitutions	70 (76)
Single	42 (46)
Tandem	11 (12)
Multiple*	17 (18)
Deletions and insertions	22 (24)
Single base deletion **	4 (4)
≥ 2 bases deletion***	16 (17)
Single base insertion	0 (0)
≥ 2 bases insertion***	2 (2)
Total plasmids sequenced	92 (100)

Plasmids having base substitutions and deletions or insertions are listed in "Deletions and insertions".

*Two had accompanying tandem base substitutions.

** Three had other base substitutions.

*** One had another base substitution.

Table 5-2 Types of base substitutions is *supF* gene in acrolein-treated plasmids replicated in normal human fibroblasts

Base substitutions	No. of base changes (%)
G:C to T:A	48 (44)
G:C to A:T	26 (24)
G:C to C:G	13 (12)
A:T to T:A	13 (12)
A:T to C:G	5 (5)
A:T to G:C	4 (4)
Total	108 (100)

The author selected *supF* mutants from experiments in which mutation frequency was at least 20-fold of the control, and DNA sequence analysis of the mutants was performed. Therefore, almost all the mutations shown here do not contain spontaneous mutations.

Mutation spectrum

Distribution of the acrolein-induced base substitutions over the coding region of the *supF* tRNA gene is shown in Figure 5-4. Mutations were not distributed randomly ($P<0.0005$) but were located mostly at specific sites. There were three hotspots (>4 base changes), at sites 133, 159 and 160, where the number of mutations observed was 4-fold or more greater than the number expected for random distribution. The most prominent hotspot was at base pair 160, where almost all base substitutions (7 of 9) were transversions. All hotspots were located at the sites of G:C base pairs.

There were 13 tandem base substitutions; five were GG to AT (or CC to AT), four were GG to TT (CC to AA). Others were one each of GA to TT (TC to AA), GC to AT, AT to TG (AT to CA) and GT to TA (AC to TA). Most of the tandem base substitutions (9 of 13) occurred at GG (or CC) sites.

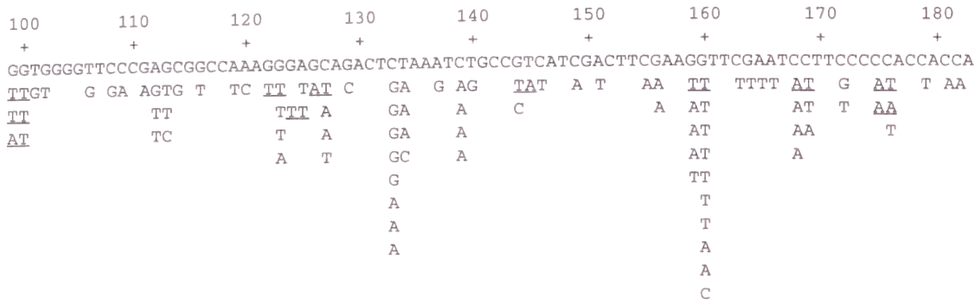


Figure 5-4. Distribution of the base substitutions in *supF* tRNA gene in acrolein-treated plasmids pMY189 replicated in normal human fibroblasts. Underlined bases are with tandem base substitutions. Sites corresponding to tRNA coding sequences (99-183) are shown. Multiple mutations, deletions and insertions are as follows (the site of the change is shown in parenthesis); G to T (32) + C to A (168) ; G to A (43) + T to A (45) ; A to T (66) + T to G (67) + G to T (160) ; C to T (80) + C to A (109) + C to T (110) ; T to G (101) + A to C (120) ; T to G (106) + C to A (149) ; A to T (112) + C to T (179) ; G to T (113) + G to T (116) ; A to T (125) + C to T (127) ; C to G (133) + C to T (146) ; C to G (133) + C to A (182) ; C to A (139) + C to A (181) ; A to T (151) + C to T (172) ; G to A (159) + C to T (163) + A to G (165) ; G to T (164) + A to T (166) ; C to A (168) + C to T (185) ; C to G (172) + C to A (176) ; 41 base pairs insertion (6-16) + G to C (129) ; deletion (16-121) ; deletion (19-122) ; deletion (30-197) ; C to T (30) + deletion (99) + G to T (102) ; deletion (42-139) ; deletion (82-183) ; deletion (94-132) ; deletion (95-127) ; deletion (96-133) ; deletion (99) + G to T (102) ; deletion (107-117) ; deletion (110-111) + C to G (114) + A to T (119) ; G to A (111) + deletion (114) ; deletion (115-212) ; deletion (125-228) ; 31 base pairs insertion (136-137) ; deletion (144) ; deletion (152-187) ; deletion (167-202) ; TTCCCC to AA (170-175) ; *supF* sequence ended at 90 bp ;

Detection of intra-strand crosslinks

Approximately one-tenth of the mutated plasmids had tandem base substitutions (Table 5-1), mainly at GG (or CC) sites (Figure 5-4), suggesting that they might have been arisen from intra-strand crosslinks. As the UV-induced pyrimidine dimers (20,21) or cis-Pt-induced intra-strand crosslinks (22) are shown to yield tandem base substitutions, the author assumed that acrolein induced intra-strand crosslinks in DNA.

Figure 5-6 shows that the 40 mer products, presumably formed by the GG intra-strand crosslinks, were detected on the lane where the acrolein-treated 20 mer substrates were loaded. No 40 mer products appeared on the lane in which CC intra-strand crosslinks might have formed. These results indicate that acrolein forms a molecular bridge between adjacent guanine bases in the same strand of DNA. The bands corresponding to the acrolein-induced inter-strand crosslinks appeared on both lanes.

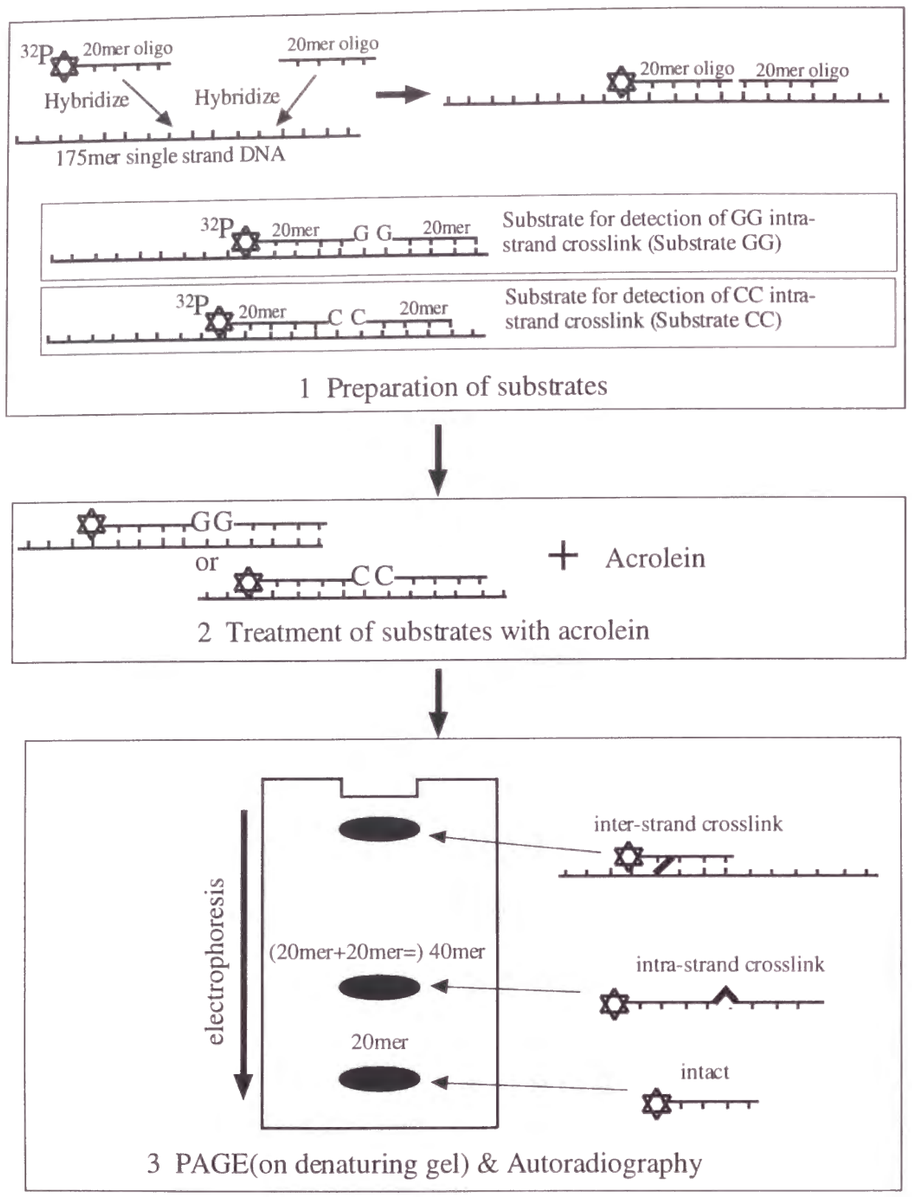


Figure 5-5. Experimental procedure for detection of the intra-strand crosslinks. 1 Preparation of substrates: A single-stranded 175 mer DNA was annealed with a ^{32}P-labeled 20 mer oligo nucleotide and a non-labeled 20 mer oligo nucleotide for an intra-strand crosslink detection. Two kinds of substrates were prepared. One is for a GG intra-strand crosslink detection and the other is for a CC intra-strand crosslink detection., 2 Treatment of substrates with acrolein: The annealed DNA was treated with acrolein., 3 PAGE & Autoradiography: DNA was separated by polyacrylamide gel (50% urea) electrophoresis. The gel was exposed to X-ray films for autoradiography.

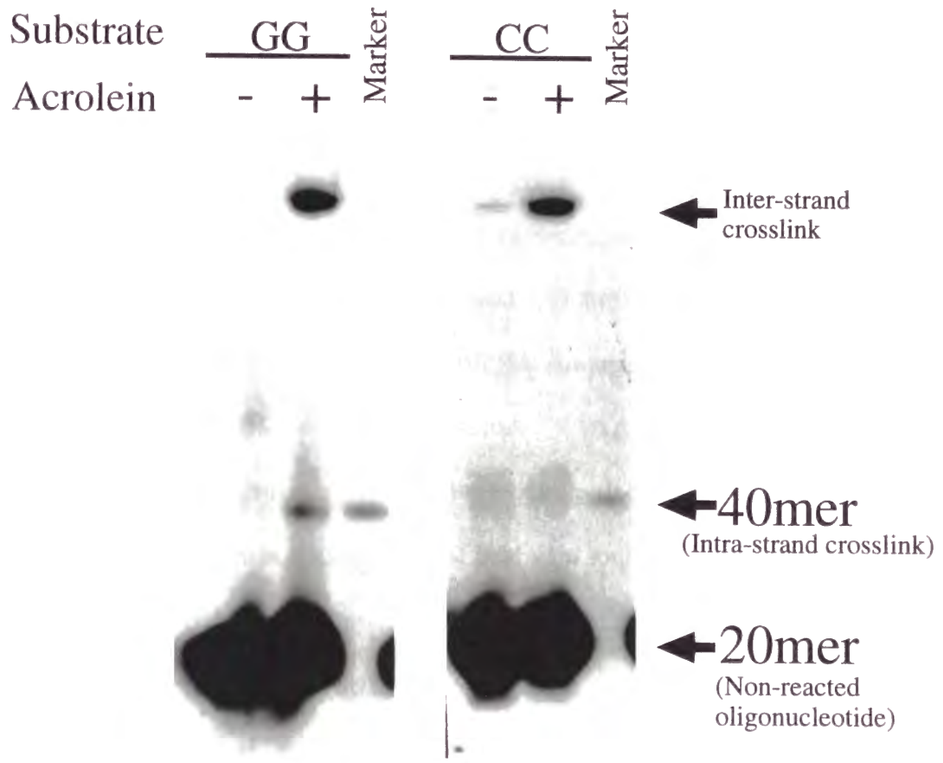


Figure 5-6. Detection of DNA intra-strand crosslinks formed by acrolein. Substrates GG and CC show substrates for detection of GG and CC intra-strand crosslink (see Figeure 5-5), respectively. Marker: 40mer single strand DNA.

Discussion

The majority (80%) of the acrolein-induced base substitutions took place at guanine or cytosine residues. These results are consistent with previous studies using PdG which has a structure similar to AdGs. The propano adducts cause G to T transversions in both mammalian and *E.coli* cells, and also G to A transitions in *E. coli* (11-13). As shown in Table 5-2, the most frequently identified base substitutions were G:C to T:A transversions and the next frequent mutations were G:C to A:T transitions. Such mutation specificity of acrolein is similar to that of PdG, possibly due to the structural similarity between PdG and AdGs.

Twenty two base substitutions (22%) were found at A:T sites. Marnett *et al.* (3) reported that acrolein induces base pair substitution mutations without the metabolic activation in *Salmonella* TA104 in which the mutational site is the nonsense sequences TAA. Acrolein produces a little amount of 1,N^6-propanpdeoxyadenosine as well as guanosine adducts(23). This propanodeoxyadenosine adduct may lead to the mutation at A:T sites.

Thirteen of the 92 mutant plasmids (14%) contained tandem base substitutions and most of these tandem base substitutions (9 of 13) occurred at GG (or CC) sites. There has been no previous report that the acrolein or propano adduct induces tandem base substitutions. It has been shown that tandem base substitutions are produced by UV(20,21) and cis-diamminedichloroplatinum(II) (cis-Pt)(22). UV induces pyrimidine dimers, and cis-Pt causes intra-strand crosslinks in DNA. The author showed that acrolein forms a molecular bridge between adjacent guanine bases in the same strand of DNA. This intra-strand crosslink might be a cause of

the tandem base substitutions which were found in the acrolein-treated *supF* gene. The molecular structure of this crosslink is not elucidated. Acrolein is a bisfunctional compound and therefore it could form a molecular bridge in DNA.

Curren *et al.* (4) reported that acrolein was strongly mutagenic to the nucleotide excision-repair deficient XP fibroblast cells, while it did not induce mutations in normal fibroblasts. Recent studies have shown that PdG could be repaired by the nucleotide excision repair system (24). As the structural difference between PdG and AdGs is presence or absence of a hydroxy group (Figure 5-1), it is unlikely that PdG and AdGs are repaired by different pathways. In our study, however, acrolein-treated plasmids pMY189 yielded the same frequency of mutations in XP cells and normal cells (Figure 5-3). Our results suggest that the major repair pathway of acrolein-induced DNA damages in human cells is not the nucleotide excision repair system.

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LIST OF PUBLICATIONS

Chapter 1

Takeji Enya, Masanobu Kawanishi, Hitomi Suzuki, Saburo Matsui and Yoshiharu Hisamatu, An unusual DNA adduct derived from the powerfully mutagenic environmental contaminant 3-nitrobenzanthrone. submitted to *Chem. Res. Toxcol.*

Chapter 2

Masanobu Kawanishi, Takeji Enya, Hitomi Suzuki, Hiraku Takebe, Saburo Matsui and Takashi Yagi, ^{32}P-Postlabelling analysis of DNA adducts formed in human hepatoma cells treated with 3-nitrobenzanthrone. submitted to *Carcinogenesis*

Chapter 3

Masanobu Kawanishi, Takeji Enya, Hitomi Suzuki, Hiraku Takebe, Saburo Matsui and Takashi Yagi, Mutagenic specificity of a derivative of 3-nitrobenzanthrone in the *supF* shuttle vector plasmids. submitted to *Chem. Res. Toxicol.*

Chapter 4

Masanobu Kawanishi, Tomonari Matsuda, Go Sasaki, Takashi Yagi, Saburo Matsui and Hiraku Takebe, (1998) A spectrum of mutations induced by crotonaldehyde in shuttle vector plasmids propagated in human cells. *Carcinogenesis*, **19** (1): 69-72

Chapter 5

Masanobu Kawanishi, Tomonari Matsuda, Aki Nakayama, Hiraku Takebe, Saburo Matsui and Takashi Yagi, Molecular analysis of mutations induced by acrolein in human fibroblast cells using *supF* shuttle vector plasmids. *Mutat. Res.* in press.

Other Publications

1. Tomonari Matsuda, Takashi Yagi, Masanobu Kawanishi, Saburo Matsui and Hiraku Takebe, (1995) Molecular analysis of mutations induced by 2-chloroacetaldeh yde, the ultimate carcinogenic form of vinyl chloride, in human cells using shuttle vectors. *Carcinogenesis*, **16** (10): 2389-2394

2. Masanobu Kawanishi, Tomonari Matsuda, Takashi Yagi, Hiraku Takebe and Saburo Matsui, (1995) DNA strand breaks in human cells induced by chlorinated organo compounds containing in Tap Water. *Environ. Mutagen Res. Co.*, **17** (2): 161-168, (in Japanese)

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