The mutagenicity of the reaction of DNA with genotoxic carcinogens

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by

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Abstract

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Mechanisms by which carcinogens induce mutations in human cells can be investigated using carcinogen exposed shuttle vector plasmids. In particular, the pSP189 plasmid can be treated with carcinogens *in vitro* and transfected into human fibroblasts. Following replication, where DNA repair or mutagenesis occurs, recovered plasmids can be screened in indicator bacteria for induced mutations in the *supF* gene.

The aim of this study was to establish and utilise assays to investigate the mutagenicity of genotoxic agents. Specifically, two model reactive intermediates of the cancer drug tamoxifen, α-acetoxytamoxifen and 4-hydroxytamoxifen quinone methide (4-OHtamQM), along with binary treatments of BPDE with UVB or UVC radiation were assessed for their mutagenic potential in human cells.

The quantitatively minor DNA adduct of tamoxifen formed by 4-OHtamQM is more mutagenic than the major tamoxifen adduct, formed by α-acetoxytamoxifen in Ad293 cells. The majority of mutations in α-acetoxytamoxifen treated plasmid were GC→TA transversions, while GC→AT transitions were predominant in 4-OHtamQM treated plasmid. Mutational hotspots were observed for both compounds. In GM04429 cells mutations induced by α-acetoxytamoxifen were mainly GC→TA, whereas in GM00637 fibroblasts GC→AT transitions were more common.

Treatment of plasmid with BPDE preferentially induced GC→TA transversions whilst UVB and UVC induced GC→AT transitions. Binary treatments were more mutagenic than single treatments, with BPDE then UV being more mutagenic than UV then BPDE. This suggests a synergistic mechanism of activation of BPDE DNA adducts by UV radiation.

In the final part of this work, a system for investigating the site-selectivity and consequences of mutagen reaction with DNA was developed. In the future this methodology can be used to detect mutations arising from specific DNA adducts inserted at known locations in important genes. This assay builds on the results provided by the standard *supF* assay and enables a more detailed investigation of the mechanisms involved in mutagenesis.

To the memory of my grandad,

Walter McLuckie

Acknowledgements

I would like to thank my supervisors Professor Peter Farmer, Professor Gordon Roberts, and Dr. Elizabeth Martin for their help and support throughout my Ph.D. project.

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Abbreviations

AAF	Acetylaminofluorene
AF	Aminofluorene
AHH	Arylhydrocarbon hydroxylases
AMS	Accelerator mass spectrometry
APCI	Atmospheric pressure chemical ionization
ATP	Adenosine triphosphate
B(*a*)P	Benzo(*a*)pyrene
BPDE	Benzo(*a*)pyrene diol epoxide
CI	Chemical ionization
CSPD	Calf spleen phosphodiesterase
dAMP	Deoxyadenosine mono phosphate
dGMP	Deoxyguanosine mono phosphate
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dG	Deoxyguanosine
dGTP	Deoxyguanosine triphosphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribose nucleic acid
dpm	Disintegrations per minute
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EI	Electron ionization
EP	Epoxide hydratase
ES	Oestrogen receptor
ESI	Electrospray Mass Spectrometry
FAB	Fast atom bombardment
FCS	Foetal Calf serum
FMN	Flavin mono oxygenase
GC	Gas chromatography
h	hour(s)
HBS	HEPES buffered saline
HEPES	(*N*- {2-Hydroxyethyl} piperazine-*N*'- [2-ethanesulfonic acid])
HPLC	High Performance Liquid Chromatography
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
HRP	Horseradish peroxidase
HSSB	Human single-stranded binding protein
IPTG	isopropyl β-D-thiogalactoside
LB-broth	Luria Bertoni broth
LC-MS	Liquid chromatography-mass spectrometry
min	minute(s)
MN	Micrococcal nuclease
NER	Nucleotide excision repair
NMR	Nuclear magnetic resonance
ODU	Optical density unit
4-OHtamQM	4-hydroxytamoxifen quinone methide

PAH	Polycyclic aromatic hydrocarbon (s)
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PEI	Polyethyleneimine
RNA	Ribose nucleic acid
s	Second(s)
supF	*Escherichia coli* tyrosine amber suppressor transfer RNA gene
TE (buffer)	tris-EDTA (buffer)
TFIIH	General transcription factor
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
Tris	(tris [Hydroxymethyl] amino methane)
UV	ultraviolet radiation
UVA	ultraviolet radiation in the range 320-400 nm
UVB	ultraviolet radiation in the range 280-320 nm
UVC	ultraviolet radiation in the range 100-280 nm
WT	Wild type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactose
XP	*Xeroderma pigmentosum*
XPA-I	Nucleotide excision repair enzymes

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Contents

Chapter 1

Introduction

1.1 Cancer in the UK

One in three people in the UK will develop cancer at some time during their life. Almost 65% of cancers are diagnosed in people over the age of 65. There are over 200 different types of cancer which can develop anywhere in the body. The four most common cancers are breast (accounting for nearly 30% of all new female cancers), lung (most common cancer in men, and third most common in women), colorectal (third most diagnosed cancer in the UK) and prostate (second most common cancer in men). Although breast cancer is the most common cancer overall, due to modern treatment and screening methods, its mortality is 8% (of all cancer caused deaths) compared to its incidence of 15%, with a 49% survival rate. Lung cancer on the other hand has a poor prognosis. The incidence of lung cancer is approximately 15% of all new cancers, but mortality is 22% of all cancer related deaths (in both men and women), with only a 5% survival rate. In 2000 there were 151,200 deaths from cancer, which is approximately one quarter of all deaths in the UK for that year (statistics from www.cancerresearchuk.org).

1.2 Carcinogenesis

Chemical carcinogens can be broadly classified as genotoxic or non-genotoxic. Genotoxic carcinogens account for over half of all reported carcinogens and initiate cancer via a direct covalent interaction with DNA. Non-genotoxic carcinogens exert effects by increasing cell proliferation or by indirectly causing DNA damage via generation of radicals.

1.2.1 Genotoxic carcinogens

There are a number of steps involved in the genotoxic carcinogenic process. One of the first stages is the formation of DNA adducts. These are formed when chemicals, or their active metabolites, react with DNA. DNA adducts can be formed following exposure to both endogenous compounds such as malondialdehyde, a product of lipid peroxidation, (Chaudhary, *et al.*, 1994; Rouzer, *et al.*, 1997), and exogenous compounds, such as benzo(*a*)pyrene (B(*a*)P) (Weinstein, *et al.*, 1976; Cosman, *et al.*, 1992).

There are three potential consequences of DNA damage: a) The DNA lesion can be repaired, returning the DNA to its natural state, b) DNA damage can lead to cell death, or c) Non repair can lead to the misreading of the DNA template in the next round of DNA replication, leading to the induction of a mutation in the gene. The most common mutation is a point mutation where a single base is replaced by another (base transitions or transversions), although insertions of bases and deletions of bases can also occur (known as frameshift mutations). A base transition is where a purine is converted to the alternate purine, or a pyrimidine is converted to the alternate pyrimidine. A base transversion is where a change between base types occurs, i.e. purine to pyrimidine. DNA mutations arise by a variety of mechanisms, including the direct miscoding of an altered base and the insertion of the wrong base opposite a non-instructional lesion. In the first scenario, the damaged base forms a base pair with the wrong base as a result of altered base pairing properties. For example, when cytosine is deaminated to uracil, the uracil can now base pair with adenine, leading to a GC→AT transition mutation. In the case of a non-instructional lesion, such as bulky aromatic DNA adducts, the adducted base is not recognised during replication.

In order to proceed with replication a random base may be inserted, most frequently adenine. This is known as the A-rule (Boiteux and Laval, 1982; Schaaper. *et al.*, 1983; Sagher and Strauss, 1983). In this way benzo(*a*)pyrene diol epoxide-guanine adducts induce GC→TA transversion mutations (Maher, *et al.*, 1989). Depending on the base involved and its location within a given region of DNA, such point mutations can have a variety of effects. These can manifest as altered peptides (caused by an altered codon), truncated peptides (caused by the generation of a stop codon) or altered regulation (caused by an alteration in signal sequences). The biological consequence of the mutation depends on the gene in question and its distribution in the cell. Accumulation of mutations in key genes such as oncogenes and tumour suppressor genes can ultimately lead to cancer (Figure 1.1).

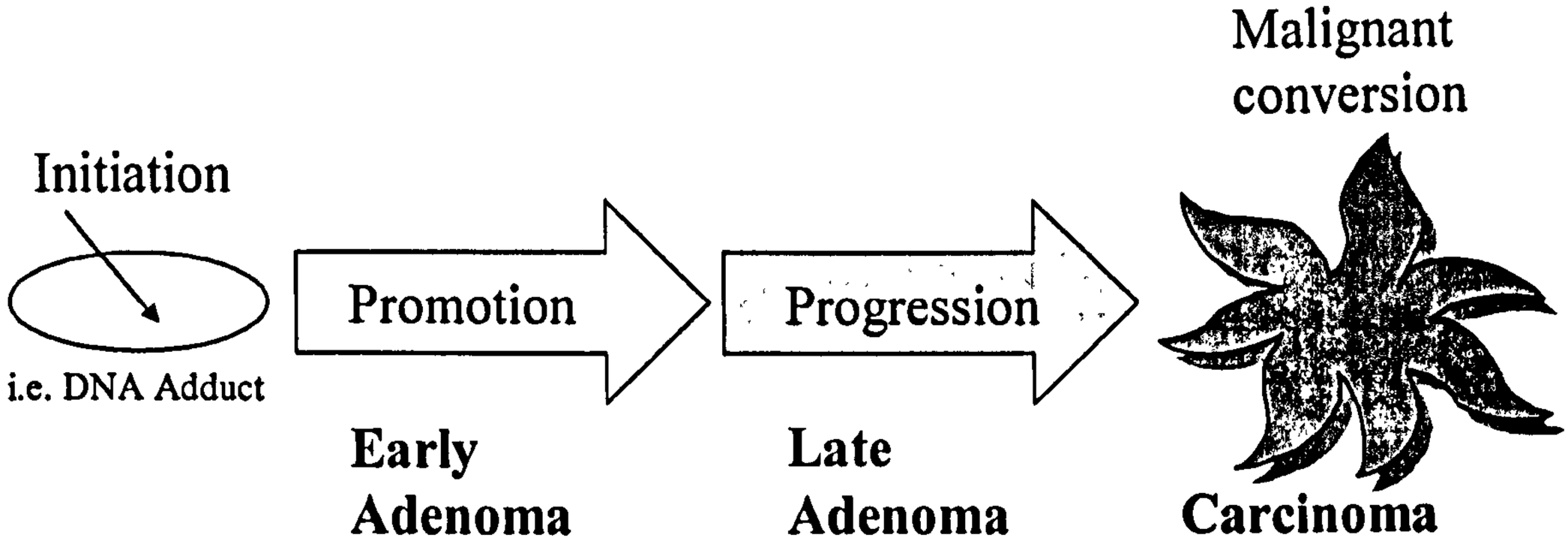


Figure 1.1 Carcinogenesis is a multistage process. Initiation by a genetic alteration, induced by DNA damage (Stage 1), is followed by promotion of any altered cells to form a benign early stage adenoma (Stage 2). If promotion is stopped adenomas will regress and disappear. If promotion is continued, then any further genetic damage can cause early adenomas to progress into late stage adenomas (Stage 3). Some of these may then undergo malignant conversion (Stage 4) and other later stages of cancer progression, such as metastasis. (Adapted from McKinnell, *et al.*, 1999)

Alteration in the p53 tumour–suppressor gene is the most common genetic defect known to occur in human cancer and is implicated in the pathogenesis of half of all human tumours (Friend, 1994). Current models suggest that wild-type p53 prevents genetic instability and is required for the apoptotic response to radiotherapy and chemotherapy (Lowe, *et al.*, 1994). It may also have a role in nucleotide excision repair as it can bind with a number of general transcription factor (TFIIH) associated proteins (Sancar, *et al.*, 1996). Studies on p53 have shown that the major mutations are transitions (such as GC→AT) (Sancar, *et al.*, 1996) although all other possible mutations have been seen. When all tumour p53 mutations are grouped together several hotspots of mutations are evident (Hollstein, *et al.*, 1991). Since endogenous and exogenous mutagens often generate specific types of base substitutions, and have preferred sites of adduction, the p53 mutational spectrum in tumours may provide information about the origins of the mutations that give rise to human cancers (Flaman, *et al.*, 1995). For example, in DNA isolated from liver tumours in areas of the world where aflatoxin B_1 (a potent genotoxic carcinogen that is produced by the food-contaminating mould *Aspergillus flavus* in warm, humid countries) exposure is common, the p53 mutation spectrum contains mainly GC→TA mutations (Ozturk, *et al.*, 1991). These mutations are known to be induced by the aflatoxin B_1-guanine adduct, as detected in the *E.coli supF* gene by Levy, *et al.*, (1992). The spectrum of mutations in the p53 gene of the liver tumours from regions where aflatoxin exposure is rare is very different. This points to aflatoxin exposure being involved in the aetiology of liver tumours in regions where exposure is high. In the same way, generation of mutation spectra from endogenous and exogenous mutagens can also be useful. Valuable information on the induction of mutations by known or suspect carcinogens can be gained by studying mutagenesis *in vitro*.

1.2.2 Non-genotoxic carcinogens

Non-genotoxic chemical carcinogens can be involved in the promotion, and progression steps of the multistep carcinogenesis mechanism (Barrett, 1993), or can, in the case of some hormones, increase the incidence of neoplasia in the absence of any initiating events (Henderson, *et al.*, 1988). It is hypothesised that oestrogens increase the frequency of mitotic activity in the breast and endometrium. Consequentially malignant phenotypes can be developed due to errors in cell division, such as DNA copying errors or chromosomal translocations (Henderson, *et al.*, 1988). Oestrogens may also induce germ cell neoplasms (testicular or ovarian cancers) by altering normal embryological development. Foetal germ cells having undergone developmental arrest, induced by oestrogens, can be stimulated to form neoplasms by gonadotrophins produced during puberty. This would suggest that oestrogens act in a two-stage model, where oestrogens act as initiators and gonadotrophins as promoters (Henderson, *et al.*, 1988). Another type of non-genotoxic carcinogens are peroxisome proliferators. This group of chemicals causes a significant increase in both the size and the number of peroxisomes in liver cells (Cattley, *et al.*, 1998). In rats and mice, this corresponds to an increase in hepatic tumour incidences (Biegel, *et al.*, 2001). The increase in number of peroxisomes increases the cellular concentration of hydrogen peroxide, which can in turn damage DNA via the formation of hydroxyl radicals. Peroxisome proliferators cause genotoxic DNA damage but through a secondary mechanism. Similarly, other chemicals which cause oxidative stress can be classed as non-genotoxic carcinogens. Asbestos workers have an increased incidence of lung carcinomas and mesotheliomas of the pleura and peritoneum (Lemen, *et al.* 1980). DNA damage due to reactive oxygen or nitrogen species has been proposed to

be involved in the molecular mechanism of asbestos-induced carcinogenicity. Accordingly, GC→TA transversions consistent with the production of 8-hydroxyguanine was shown to be induced by asbestos in the *lacI* gene of rats (Unfried, *et al.*, 2002).

1.3 DNA damage

DNA adducts are a type of DNA damage, arising when a compound, which is either itself reactive or is metabolised to an electrophilic species, covalently binds to nucleophilic sites on DNA. DNA adducts are considered an early initiating event in the carcinogenic process since if they are not repaired correctly before DNA replication, they may lead to incorporation of an incorrect base resulting in a mutation. Along with mutations induced by DNA adducts, there is also the possibility of mutations arising from spontaneous chemical alterations in DNA. If mutations are fixed and present in critical genes such as tumour suppressors or proto-oncogenes, then they may ultimately result in cancer (Barrett, 1993). Other carcinogens can induce the fixation of mutations by interfering with the cells ability to process DNA damage. For example, cadmium is classed as a carcinogen as it is thought to inhibit the DNA repair of spontaneous DNA lesions. It can inhibit the ability of O^6-methylguanine methyl transferase (Sicchitano and Pegg, 1987), inhibit removal of oxidative DNA damage from human cells (Dally and Hartwig, 1997) and can inhibit lesion recognition in nucleotide excision repair, in human cell-free extracts (Calsou, *et al.*, 1996). The presence of DNA adducts is therefore an indication that a drug may be carcinogenic.

1.3.1 Spontaneous alterations in the chemistry of DNA bases

1.3.1.1 Tautomeric shifts

Each of the four common bases in DNA can undergo a transient rearrangement of structure, termed a tautomeric shift, to form a structural isomer of the base (tautomer) (Watson, 1976). Formation of tautomers alters the base pairing ability of the bases. The exocyclic amino (NH_2) groups of adenine, guanine and cytosine can tautomerise to imino (NH) groups. When either adenine or cytosine is in this latter configuration, it can mispair with the other through the two available hydrogen bonds leading to AC basepairs rather than GC. Similarly both guanine and thymine can tautomerise at the position of the C6 oxygen. The usual keto (C=O) group can be converted to an enol (C-OH) group. This means that base mis-pairing can also occur between guanine and thymine utilising the three available hydrogen bonds giving TG rather than TA (Cadet and Berger, 1985). If any base in a template strand is in its rare tautomeric form then misincorporation in the daughter strand can occur during DNA replication. Other mispairing schemes have also been suggested (Drake, 1991). Switching of the base to the *syn* configuration (180° rotation around the glycosidic bond) or the inclusion of a water molecule as a bridge may also be possible.

1.3.1.2 Deamination of bases

Three of the four bases normally found in DNA have exocyclic amino groups (adenine, guanine and cytosine). The loss of these groups occurs spontaneously in pH- and temperature- dependent reactions of DNA (Lindahl, 1979; Lindahl, 1993; Shapiro, 1981). This results in the formation of hypoxanthine, xanthine and uracil, respectively, which can give rise to mutations during DNA replication (Lindahl,

1974). Hypoxanthine can base pair with cytosine, resulting in AT→GC transition mutations during DNA replication. Xanthine is unable to pair stably with either cytosine or thymine and arrest of DNA synthesis may occur on templates containing this lesion. Uracil will base pair with adenine during DNA replication, resulting in GC→AT transition mutations.

1.3.1.3 Loss of bases - depurination and depyrimidination

The loss of purines and pyrimidines from DNA has been extensively studied at acid pH, but loss of purines and pyrimidines can also happen at neutral or basic pH (Lindahl, 1993; Loeb and Preston, 1986). The rate of depurination and depyrimidination of double and single stranded DNA can be observed by incubating DNA at various temperatures and pHs and measuring the rate of release of labelled bases (Lindahl and Nyberg, 1974). Guanine was found to be released around 1.5 fold faster than adenine at both acidic and neutral pH, but at alkaline pH dAMP is hydrolysed more rapidly than dGMP (Lindahl and Nyberg, 1974). At acidic pH depurination was also found to occur more rapidly than depyrimidination (Shapiro, 1981). The chemical mechanism of hydrolytic DNA depurination at acidic pH is believed to be the same as that established for acid hydrolysis of deoxynucleosides, i.e. the base is protonated followed by direct cleavage of the glycosyl bond (Lindahl and Nyberg, 1972; Zoltewicz, *et al.*, 1970). The mechanism for alkaline and neutral hydrolysis of nucleosides is much less well characterised (Lindahl, 1979). From the rate measurements at high temperatures and extrapolation of this data, the rate of depurination of double stranded DNA at physiological pH, temperature and ionic strength has been calculated as 3 depurinations per 10^{11} purines per second (Lindahl and Nyberg, 1972; Zoltewicz, *et al.*, 1970). This corresponds *in vivo* to

approximately one purine per *E.coli* genome per generation, given the DNA doubling time of 1 hour (Lindahl, 1979), or for mammalian cells, which have larger genomes and longer replication times, this corresponds to a purine loss rate of 10,000 per cell generation (Lindahl, 1979). Pyrimidine nucleosides are more stable than purine nucleosides. The mechanism of cleavage at the glycosyl linkage is the same as with purines but cytosine and thymine are lost at rates of only 1/20 that observed for the purines (Lindahl and Karlström, 1973). The deoxyribose residues left behind on the DNA backbone after depurination and depyrimidination can undergo rearrangement, to an open aldehyde form rather than the closed furanose form, and can be further hydrolysed by a β-elimination reaction (Jones, *et al.*, 1968) resulting in DNA strand breaks 3' to the apurinic/ apyrimidinic site.

1.3.1.4 Oxidative damage to DNA

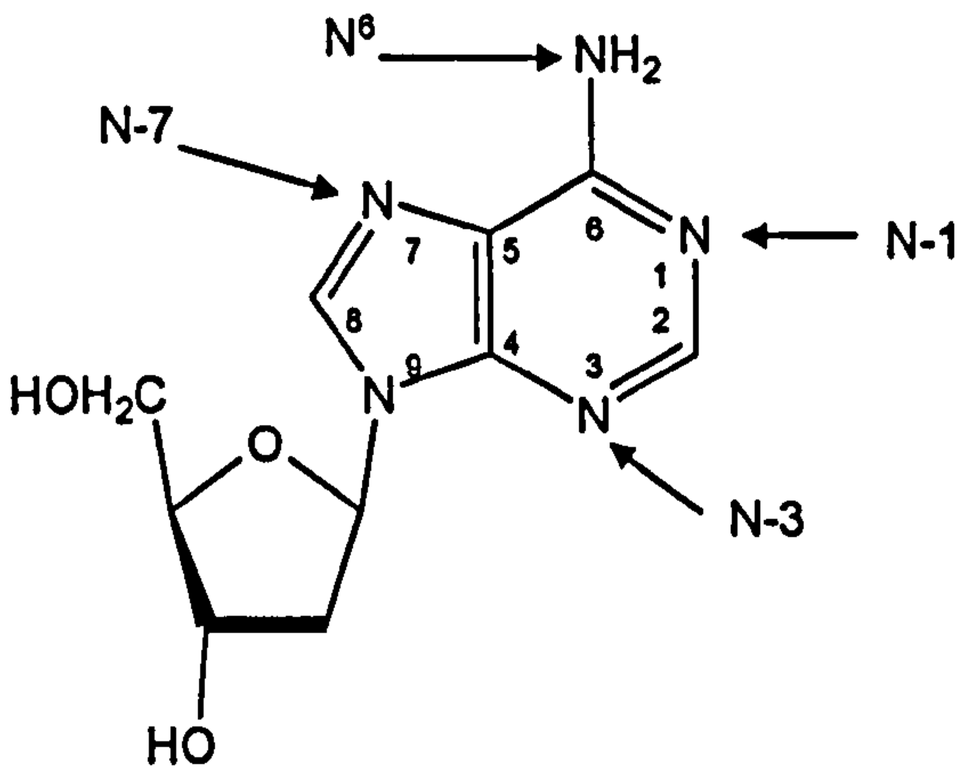
Attack of DNA by reactive oxygen species must be considered as one of the major sources of spontaneous DNA damage. They also play an important role in radiation induced DNA damage (see later). There are various intra- and extra-cellular sources of oxygen radicals (Clayson, et al., 1994; Saran and Bors, 1990). The major source of oxygen radicals is probably leakage associated with the reduction of oxygen to water during mitochondrial respiration. These products are singlet oxygen, peroxide radicals ($\cdot O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\cdot OH$). Other intracellular processes that result in the release of reactive oxygen include peroxisomal metabolism, the enzymic synthesis of nitric oxide, and the metabolism of phagocytic leukocytes (Badwey and Karnovsky, 1986). Extracellular sources of reactive oxygen species include ionizing radiation, UV light (especially in 320-380 nm region, UVA), heat, various drugs, and redox cycling compounds. These oxygen

radicals can also abstract electrons from residues of important organic macromolecules which can result in the initiation of chain reactions and damage at considerable distances from the original chemical event i.e. at opposite ends of a cell (Saran and Bors, 1990). One such example of a chain reaction is the peroxidation of unsaturated lipids initiated by reactive free radicals such as ·OH (Vaca, *et al.*, 1988). The half-life of these generated radicals is important in their potential for DNA damage, as is their diffusibility. Peroxyl (ROO·) and alkoxyl (RO·) radicals are likely intermediates in many chain reactions, whilst peroxides (ROOH and H_2O_2), alcohols (ROH) and carbonyls (RHC=O) are likely end products. Hydrogen peroxide (H_2O_2) is probably the most significant of these products as, although it is relatively inert, it can form·OH radicals in a process catalysed by transition metal ions, typically Fe^{2+}, known as the Fenton reaction (Fenton, 1894), potentially resulting in DNA damage (Carmichael, *et al.*, 1992). Since hydrogen peroxide has a long diffusion range hydroxyl radicals can cause damage a fair distance away from the original chemical insult.

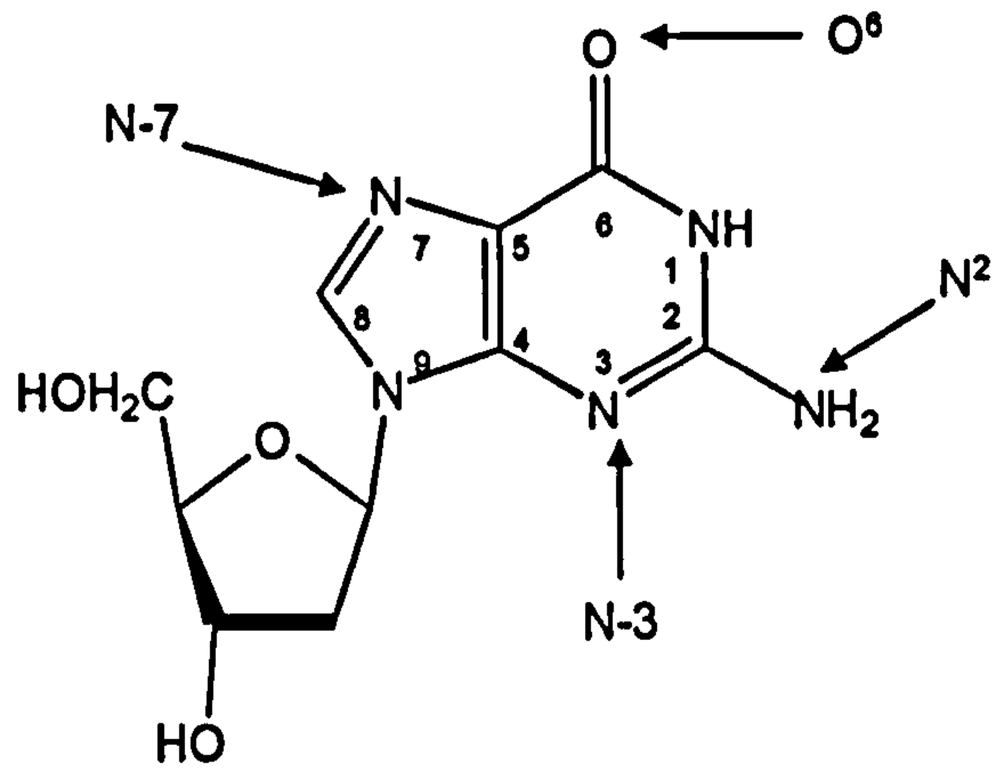
1.3.2 Alterations in DNA bases caused by environmental damage

Combined with the endogenous sources of damage, DNA is also prone to attack from exogenous carcinogenic and mutagenic compounds from the environment. These can be in the form of DNA reactive compounds found in food and drink, from automotive exhaust emissions, cigarette smoke, both through direct and passive smoking, in the air, in medicinal drugs or from the administration of high energy radiation in the form

of ionizing radiation, and ultraviolet light. Figure 1.2 illustrates the possible sites of DNA base modification from genotoxic carcinogens.



2'-Deoxyadenosine



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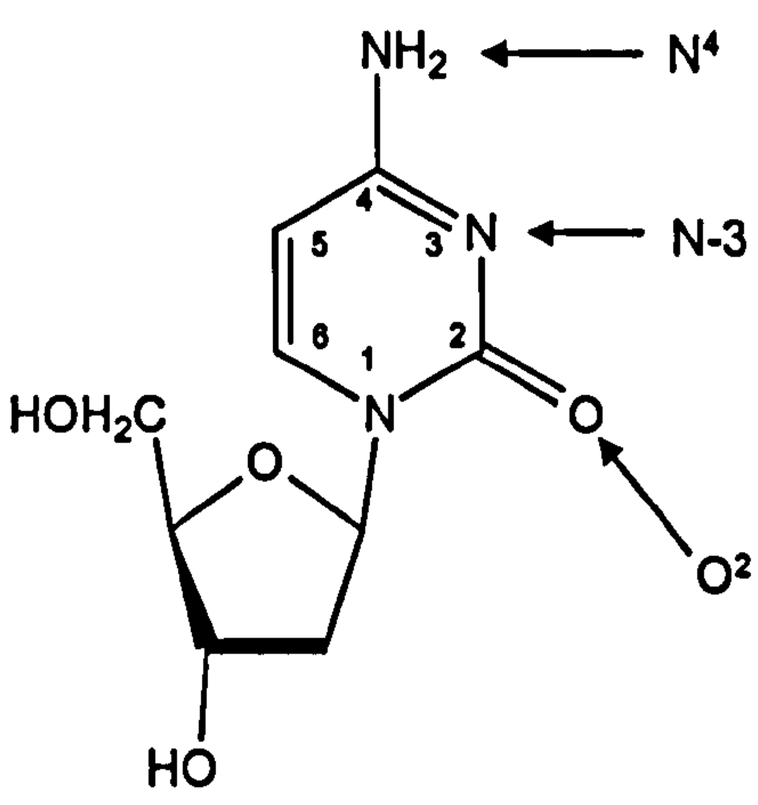
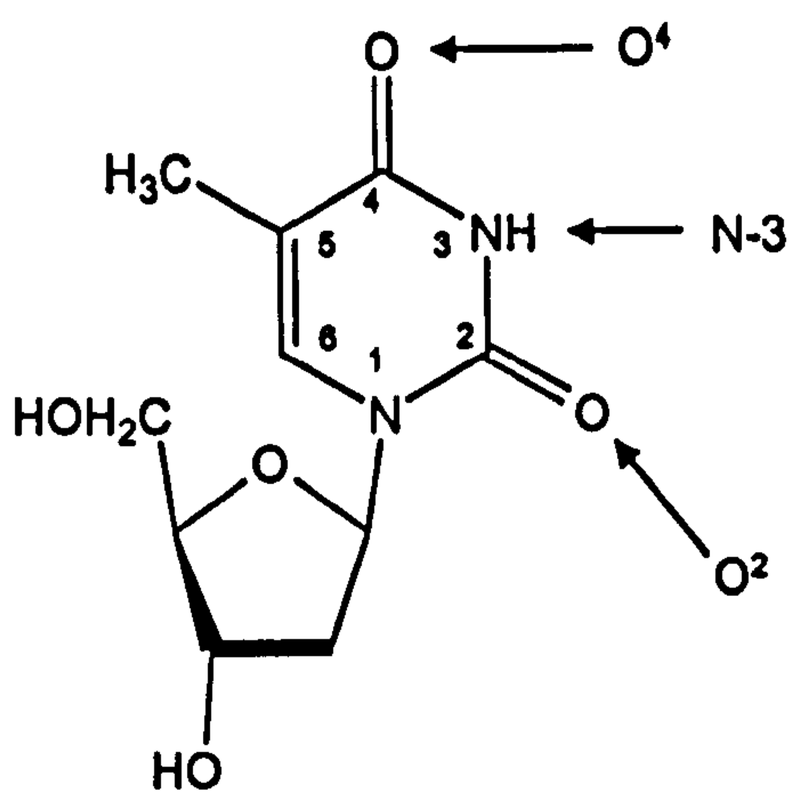


2'-Deoxycytidine



2'-Deoxythymidine

Figure 1.2 The potential sites for modification of DNA bases.

1.3.2.1 Chemical carcinogens

1.3.2.1.1 Organic compounds

Organic compounds can be subdivided based on the chemistry of the donor groups which are transferred to DNA bases. These are i. alkylating agents, ii. Aralkylating agents, and iii. Arylamines.

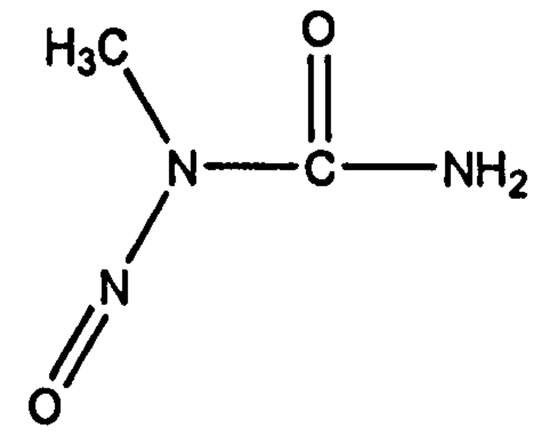
Alkylating agents

Alkylating agents are chemicals that transfer alkyl groups, often methyl or ethyl groups, to nucleotides to form DNA adducts. The *N*-nitroso compounds, especially nitrosamines, are perhaps the most hazardous of the various types of alkylating carcinogens (O'Neill, *et al.*, 1991). Epidemiological studies have provided no definite link between members of this class of carcinogens and human cancer, although chloroethylnitrosureas were used as chemotherapeutic agents for gastrointestinal cancers (Boice *et al.*, 1983) resulting in some cases of leukaemia. The group of tobacco-specific nitrosamines are found in tobacco and tobacco smoke (O'Neill, *et al.*, 1991). The mechanism by which smoking causes lung cancer is, however, complex and can also involve the production of polycyclic aromatic hydrocarbons (PAH). Several of these *N*-nitroso compounds have been found to be carcinogenic in every species tested, including non-human primates (e.g. Magee and Barnes, 1956; Kelly, *et al.*, 1966). These findings along with the hepatotoxicity associated with occupational exposure to nitrosamines (Kimbrough, 1983) and the ubiquitous nature and volatility of these chemicals strongly suggest that they pose a significant health hazard to humans (Bartsch, *et al.*, 1992).

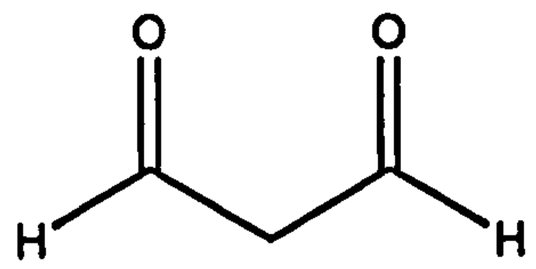
Activation of these compounds often requires biotransformation either by enzymic oxidation (such as for *N*-nitrosodimethylamine) or directly by alkali-mediated hydrolysis (such as for the direct-acting carcinogen *N*-methyl-*N*-nitrosourea) (Figures 1.3 & 1.4). Either way a methyl group (CH_3-) or other alkyl group (R-), depending on the chemical, is available to modify a DNA base. Alkylation occurs predominantly at exocyclic oxygens and nitrogens or on ring nitrogens. Although these classes of carcinogens are distributed throughout the environment, they can also be formed

endogenously. *N*-Nitroso compounds can be formed by the nitrosation of secondary amines under acidic conditions, such as in the stomach, whilst gut fauna can catalyse similar reactions under physiological, non acidic, conditions. Whether these endogenous sources of alkylating agents represent a significant health risk has not been established.

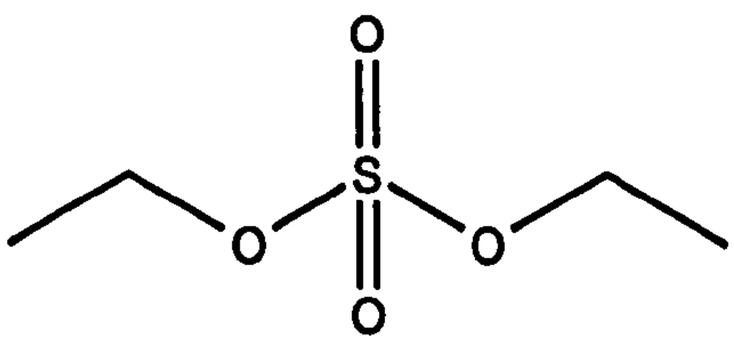
Direct acting alkylating agents



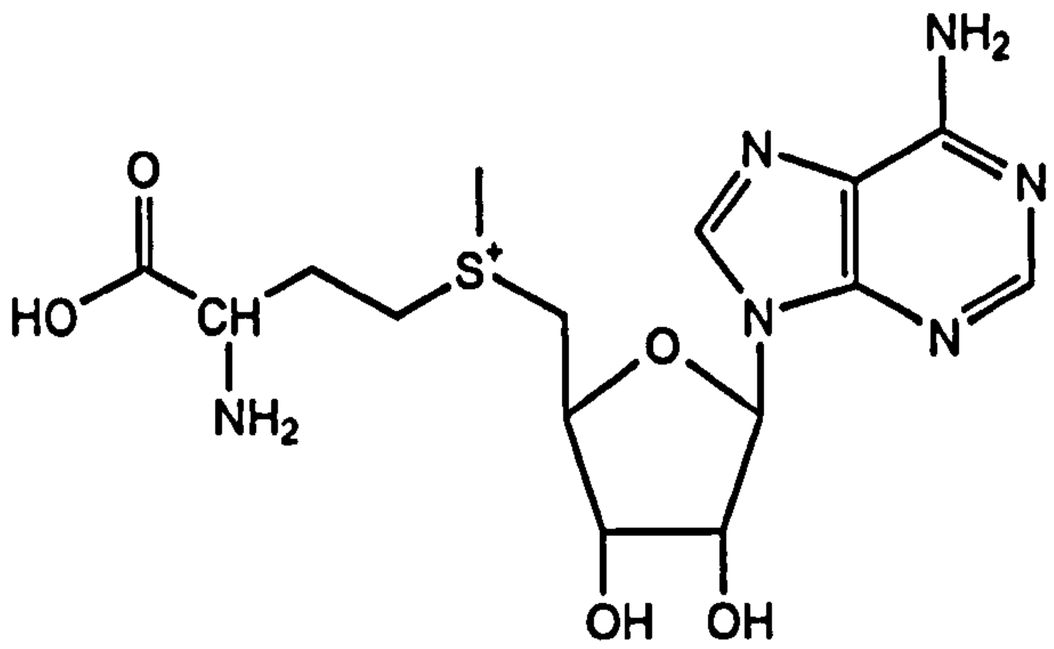
N-Methyl-*N*-nitrosourea



Malondialdehyde



Diethyl sulphate



s-Adenosyl-methionine

Figure 1.3. Some examples of direct acting alkylating agents.

Other alkylating agents can contain oxygen, such as malondialdehyde (Figure 1.3), a by-product of arachidonic acid metabolism, or sulphur, such as diethylsulphate or *s*-adenosyl-methionine, as their electron withdrawing groups (Figure 1.3). Other important alkylating agents also have to be metabolised before they bind with DNA, such as the group of aflatoxins, naturally produced by the mould *Aspergillus flavus*.

Activated alkylating agents

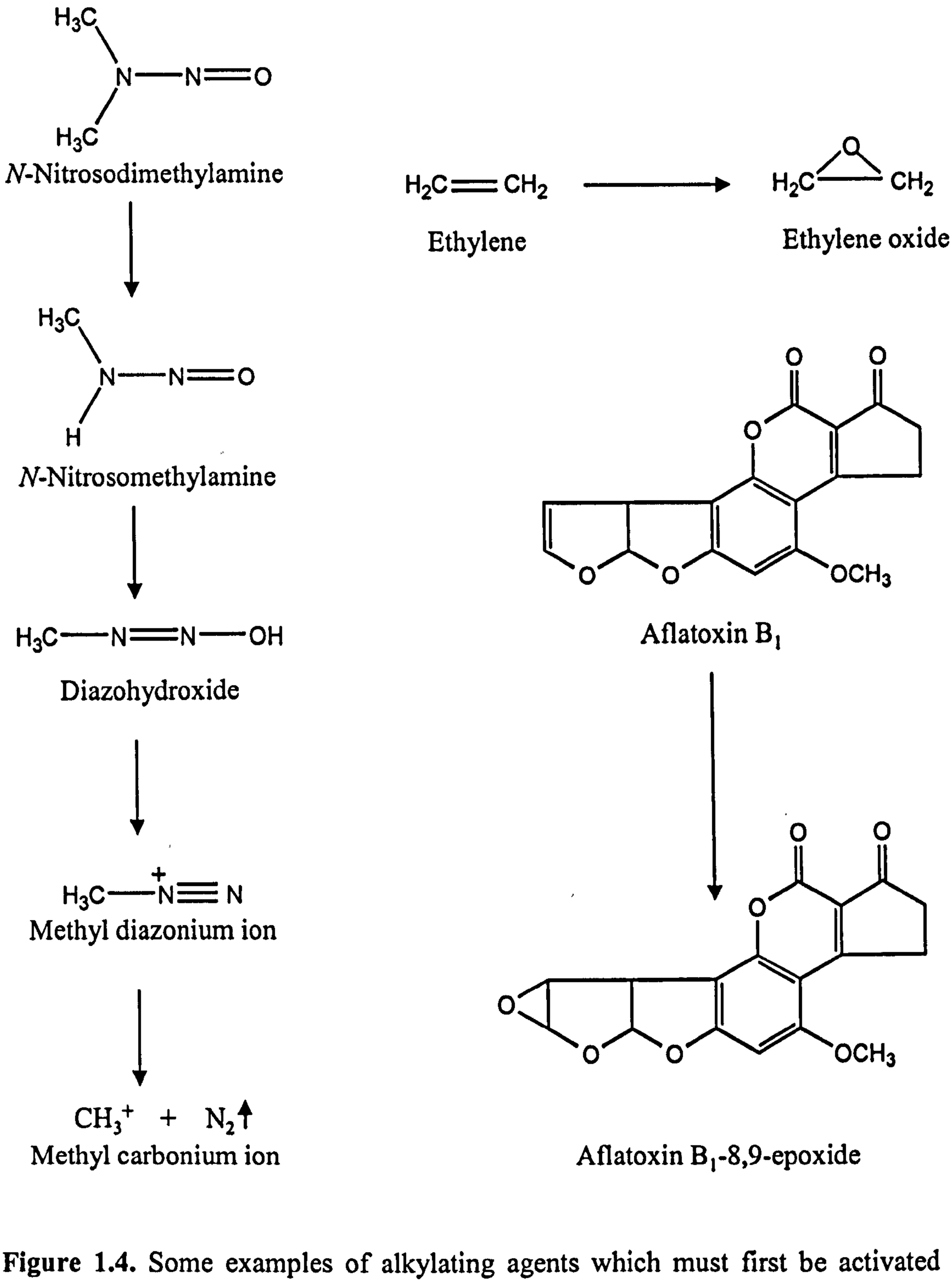


Figure 1.4. Some examples of alkylating agents which must first be activated by members of the cytochrome P450 mixed function oxidase group of enzymes.

Aflatoxin B_1, the best studied of these compounds, is metabolised to a reactive epoxide (Figure 1.4) which binds with DNA. Aflatoxin B_1 has been shown to be a

carcinogen in many animal species (Busby and Wogan, 1984) and causes human hepatocellular carcinoma in Asia and Africa (Hollstein, *et al.*, 1991; Ozturk, *et al.*, 1991). Epoxides are an important class of alkylating agents which occur due to metabolism of both exogenous and endogenous compounds. For example, the important industrial chemical ethylene, which is also formed endogenously, is metabolised to ethylene oxide which forms DNA adducts (Li, *et al.*, 1992; van Sittert, *et al.*, 2000) (Figure 1.4).

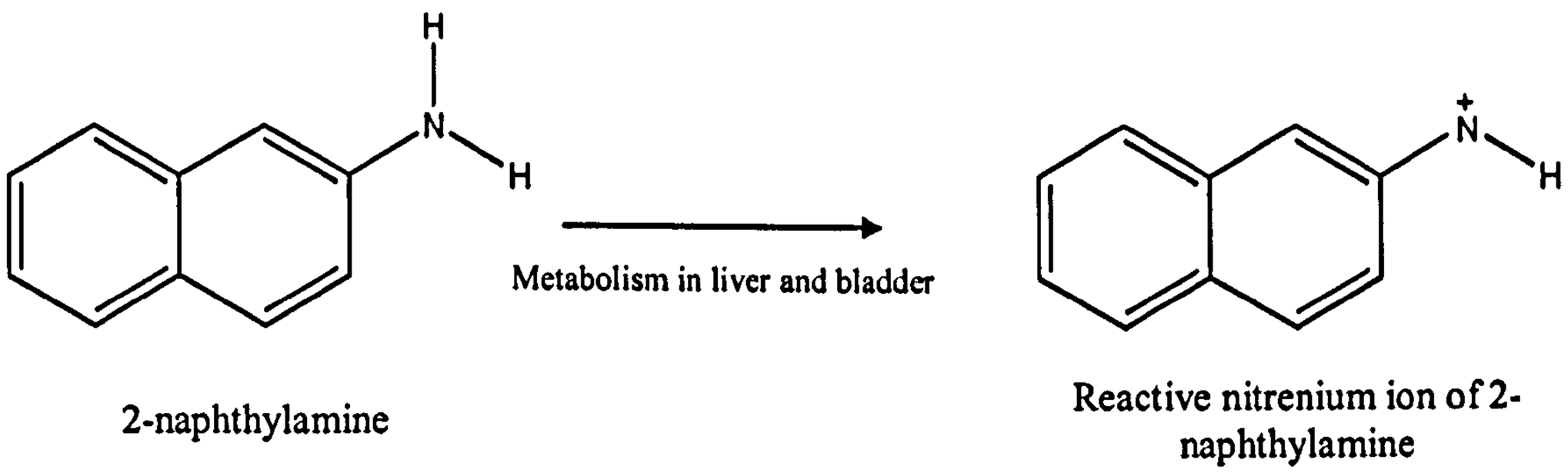
Aralkylating agents

Aralkylating agents are chemicals that transfer aromatic groups to nucleotides to form DNA adducts. The principal group of aralkylating compounds, the polycyclic aromatic hydrocarbons (PAH), are formed in combustion products from automotive engines (Grimmer, *et al.*, 1983), in crude oils, cigarette smoke and also in charcoal grilled meats (Lijinsky and Shubik, 1964). Benzo(*a*)pyrene is one of the most studied polycyclic aromatic hydrocarbons and is described in full in Chapter 3. The breast cancer drug tamoxifen forms DNA adducts and increases the risk of endometrial tumours in treated women. It is presently not known whether this is due to a genotoxic mechanism. Tamoxifen is described in full in Chapter 4.

Arylamines

Arylamines are chemicals that transfer aromatic amines to nucleotides to form DNA adducts. The carcinogenicity of aromatic amines was established after epidemiological studies of workers from the dye industry. It was found that a high incidence of bladder cancer could be attributed to occupational exposure of aniline dyes (Rehn, 1895). The agents most responsible for this cancer were later found to be

2-naphthylamine and benzidine (Case, *et al.*, 1954). Arylamines require metabolism by cytochrome P450 mixed function oxidase enzymes, and generally undergo *N*-oxidation to generate reactive intermediates. For example, 2-naphthylamine is first enzymically oxidised by arylhydrocarbon hydroxylase (AHH) to an *N*-hydroxyl intermediate, which is further metabolised in the liver or kidney to form a stable glucuronide conjugate that is transported to the bladder (Kadlubar, *et al.*, 1977). The ultimate carcinogenic form, the electrophilic nitrenium cation species (Figure 1.5), is formed under the acid conditions of the bladder, where it can react with DNA in bladder epithelial cells (Orzechowski, *et al.*, 1992).



2-naphthylamine

Reactive nitrenium ion of 2-naphthylamine

Figure 1.5 2-naphthylamine, used in the dye industry, is metabolised to form a carcinogenic nitrenium ion

1.3.2.2 Ionising radiation

Ionising radiation was first recognised as a human carcinogen during the development of Roentgen's cathode tube, the basis for the X-ray machine. Early radiologists used to focus the electron beam using their own hands, resulting in the frequent induction of skin cancer (Frieben, 1902). Luminescent dial painters in watch factories suffered a high incidence of osteosarcomas (Martland, 1931). Painters ingested radium when they used their mouths to form pointed tips on their paintbrushes. The radium was

localised to the bone due to its similarity to calcium, making bone the immediate target for high energy alpha emissions. After World War II atomic bomb survivors in and around Nagasaki and Hiroshima had an increased risk of cancer mortality, especially from leukaemias (Shimizu, *et al.*, 1989 & 1990).

The mechanism of carcinogenesis from ionising radiation is believed to involve indirect formation of mutagenic oxygen free radicals. Due to the tissue penetration of certain types of ionising radiation, oxygen radicals can be generated when the water molecules surrounding DNA are ionised. Once formed the reactive oxygen species can induce more than thirty different types of DNA adduct, e.g. 8-hydroxyguanine, thymine glycol, 8-5'-cyclodeoxyguanosine, as well as DNA-protein cross links (Feig, *et al.*, 1994).

1.3.2.3 Ultraviolet radiation

Ultraviolet radiation has been shown to be very important in the induction of skin cancer (Urbach, 1993; Marks, 1996). The action of ultraviolet radiation in respect to DNA damage is further detailed in Chapter 2.

1.4 DNA repair

Since any DNA damage caused by endogenous and exogenous origins can be potentially deadly to the cell and in some cases to the whole organism, a complex system of damage detection and repair has evolved. DNA repair occurs in the cell nucleus immediately prior to replication. The p53 protein is recruited, along with the appropriate repair proteins, to the site of damage. The increased concentration of p53

in the area triggers termination of the G_1 phase and the initiation of the S phase in which replication of the newly repaired DNA occurs. Any unrepaired lesions may result in the incorporation of mismatch bases. The four main human systems are summarised below.

1.4.1 Direct repair

The direct repair of DNA damage involves breakage of the abnormal chemical bonds between bases by specific enzymes. An example found in humans is the enzyme O^6-methylguanine DNA methyl transferase. This enzyme transfers the methyl group of O^6-methylguanine to a cysteine residue present on the enzyme yielding the unmodified guanine in DNA. This is a 'suicide process' as the protein is now inactivated by the methyl group. The enzyme can also transfer other alkyl groups but with less efficiency (Miller, 1983)

1.4.2 Transcription coupled repair

Transcribed DNA is repaired faster than non-transcribed DNA in humans (Chen, *et al.*, 1990; Inga, *et al.*, 1994). There is also a preference for genes transcribed by RNA polymerase II. Chromatin structure, the topology of the transcribed DNA, and the effects of lesions in the path of RNA polymerases all contribute to this effect. Bulky lesions such as dipyrimidine dimers will stop transcription if present on the template strand but not if present on the coding strand. A complex of two or more proteins displaces the RNA polymerase once it is caught up at the lesion, allowing for excision nucleases to bind in its place.

1.4.3 Base excision repair

Base excision repair is carried out by the DNA glycosylase class of enzymes. Enzymes in this group recognise a specific abnormality in the DNA: such as damage from alkylating agents, inappropriate bases such as uracil in DNA, or mispaired bases. DNA glycosylases catalyse the hydrolysis of *N*-glycosyl bonds; the bonds linking the bases to the deoxyribose-phosphate backbone of DNA. The loss of bases results in abasic sites. These are recognised by apurinic/apyrimidinic endonucleases resulting in hydrolysis of the phosphodiester bonds 5' to the abasic site. DNA polymerases are now free to bridge the gap, using the correct template strands as primers (Bohr, *et al.*, 1985).

1.4.4 General nucleotide excision repair

The interaction of DNA with physical or chemical insults can cause a wide range of base damage. Nucleotide excision repair (NER) pathways act on these various anomalies; such as simple methylation of bases through to more complex covalently joined pyrimidine dimers or 6-4' photoproducts. NER pathways tend to work more efficiently if the DNA helical structure is altered, i.e. by the presence of bulky adducts. The enzymes involved in NER were first discovered in cells of patients with *Xeroderma pigmentosum* (XP). XP is a genetic disease in which sufferers have a high incidence of skin cancers in areas of their bodies exposed to the sun (Cleaver and Kraemer, 1989; Kraemer, *et al.*, 1987). Fibroblast cells derived from these patients are deficient in excision repair enzymes, which are required for the repair of certain types of DNA damage. UV induced dipyrimidine dimers and bulky DNA adducts are not repaired correctly whereas strand breaks or damage caused by ionizing radiation

are repaired normally. This is why the polypeptides involved in human nucleotide excision repair are denoted XPA, XPB, and so on.

There are at least 17 polypeptides involved in the human excision repair mechanism. Damage recognition protein (XPA) binds to the heterodimer of the two polypeptides XPF and ERCC1 (Freidberg, 1996). Which in turn bind to the replication protein HSSB. This four member complex binds to the site of DNA damage as XPA binds damaged DNA in preference to undamaged DNA. General transcription factor TFIIH is now recruited to the site of damage by XPA. The TFIIH complex is made up from eight subunits, two of which (XPB and XPD) have helicase activity (Sancar, 1994; Schaeffer, *et al.,* 1993), along with two more (XPC and XPG) which are either loosely associated with TF11H or are recruited to the complex through interactions with TF11H. The conformational change brought about by the unwinding of the DNA double helix by XPB and XPD allows the two proteins with nuclease activity, XPF and XPG, to make dual incisions 5' and 3' respectively to the lesion. In humans the 3' nick-lesion distance is three or four nucleotides whilst the 5' nick is situated about twenty one nucleotides away from the lesion (Friedberg, 1996). After incision a subset of the complex remains bound to the DNA. Catalytic turnover of the enzyme complex is facilitated by PCNA (proliferating cell nuclear antigen) allowing release of the oligomer along with the enzymic subunits. The excision gap is filled by DNA polymerases polδ and polε followed by ligation to form the completed DNA strand. An overview of this process is illustrated in Figure 1.6. Any missed lesions can result in the incorporation of mismatch bases during replication. These may then either be repaired by mismatch repair enzymes or if unrepaired could pass the mutation on to future cell generations.

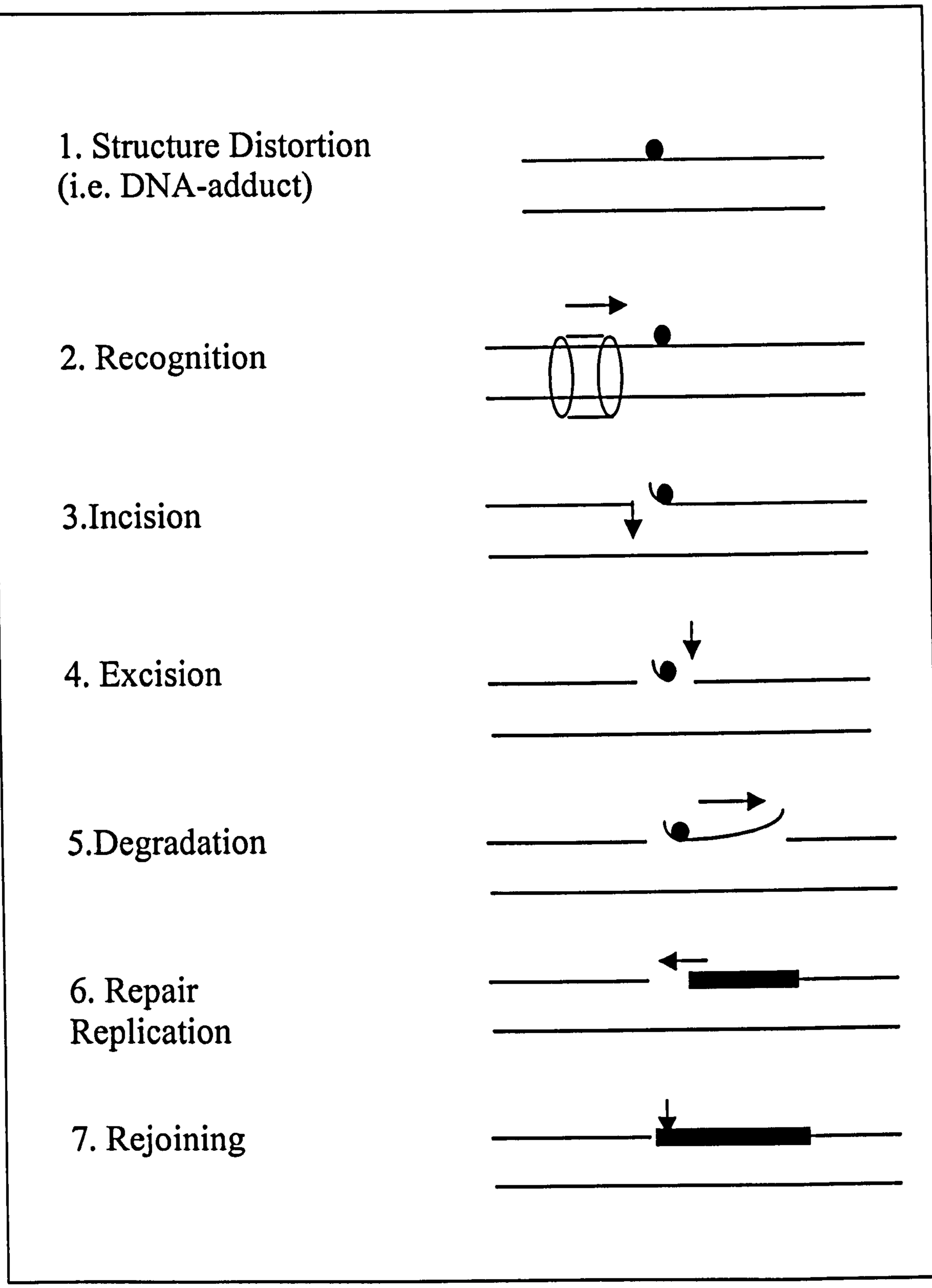


Figure 1.6 An overview of Nucleotide Excision Repair

1.5 Genetic diseases with a predisposition to cancer

If DNA damage cannot be repaired, then cells become more susceptible to the induction of cancer. Some genetic diseases have mutant or missing DNA repair genes, such as XP (Chu and Mayne, 1996), Cockaynes syndrome (Cockayne, 1936) and Trichothiodystrophy (Pollitt, *et al.*, 1968). Other patients who are predisposed to cancer have mutations in genes involved at a later stage of cancer progression, such as those related to proliferation, differentiation, or apoptosis. Patients with familial adenomatous polyposis (FAP) have a mutation in the APC (adenomatous polyposis coli) cancer suppressor gene (Fodde, 2002). Patients lacking the APC gene are likely to develop colorectal cancer by the age of 40. Likewise, mutations in the *BRCA1*/BRCA2 tumour-suppressor genes are linked to familial incidences of breast cancer (Blackwood and Weber, 1998).

1.5.1 *Xeroderma pigmentosum*

XP is a rare autosomal recessive disease. Affected patients are sensitive to the sun, resulting in progressive degenerative changes to sun exposed portions of the skin and eyes, often leading to neoplasia. Some XP patients also develop progressive neurologic degeneration. Symptoms are generally only seen in homozygotes.

1.5.1.1 History

Historically XP was first observed and documented in 1863 by Moritz Kaposi. His term Xeroderma, or parchment skin, was later reported in the dermatology textbook co-written with Ferdinand von Hebra (1874). In 1883 the term pigmentosum was added to emphasise the striking pigmentary abnormalities. In 1968 James Cleaver reported that defective DNA excision repair was seen after cultured skin fibroblasts

from XP patients were irradiated with ultraviolet light. This was further demonstrated *in vivo* by Epstein *et al. in* 1970. Burk, *et al.* (1971) described a patient with XP who exhibited normal excision repair. This was subsequently named *variant* XP (Cleaver, 1972), although it was later found to have an abnormality in another DNA repair system, post-replication repair (Lehmann, *et al.* 1975, Cleaver, *et al.*, 1980). In 1972, De Weerd-Kastelein, *et al.* demonstrated genetic heterogeneity in the DNA excision repair defect of XP by using cell fusion techniques. Fibroblasts from two XP patients were fused to form a heterokaryon (a cell with two nuclei in a common cytoplasm) which exhibited mutual correction (or complementation) of the defects in DNA repair. Each cell supplied what the other was lacking, implying that each cell had different excision repair defects. These cells are said to be in different complementation groups. The complementation groups along with their relative rates of DNA repair are denoted in Table 1.1.

Table 1.1 *Xeroderm pigmentosum* (XP) complementation groups, their relative frequencies of incidence and repair efficiencies relative to normal cells.

Complementation Group	Incidence Frequency (%)[a]	Percent of normal repair	Gene mutated	Clinical form	Reference
A	29.4	2 - 5	*XPA*	Neurologic	Kraemer, *et al.*, 1975
B	0.5	3 - 7	*XPB*	Neurologic	Kraemer, *et al.*, 1975
C	27.3	10 - 20	*XPC*	Classic	Kraemer, *et al.*, 1975
D	15	5 - 15	*XPD*	Neurologic	Kraemer, *et al.*, 1975
E	1.1	25 - 50	*XPE*	Classic	Kraemer, *et al.*, 1975
F	1.6	40 - 50	*XPF*	Classic	Arase, *et al.*, 1979
G	1.1	18	*XPG*	Neurologic	Keijzer, *et al.*, 1979
H	0.5	< 2	*XPH*	Neurologic	Moshel, *et al.*, 1983
I	0.5	30	*XPI*[b]	Neurologic	Fischer, *et al.*, 1985
Variant	24.1	15 - 40	*XPV*[c]	Neurologic	Cleaver, 1972

[a] from total of 189 patients. (Kraemer, *et al.*, 1987)

[b] It is not sure what this gene product does in DNA repair.

[c] XPV gene not yet cloned and characterised.

1.5.1.2 Epidemiology and symptomatology

XP has been found in all races worldwide. The frequency is about 1 in 250,000 in the United States and Europe but is considerably higher in Japan (1 in 40,000) and Egypt. In a literature survey of more than 800 patients there were nearly equal numbers of males and females (54% and 46%, respectively) (Kraemer, *et al.*, 1987). Nearly 20% of the patients had neurologic abnormalities, including a large proportion of Japanese patients. The median age of onset of symptoms is between 1 and 2 years, although in 5% of patients the onset is delayed until after 14 years (Kraemer, *et al.*, 1987). The cutaneous, ocular and neurological symptoms of XP are summarized in Table 1.2 (Kraemer, *et al.*, 1987).

Table 1.2 Cutaneous, ocular and neurological abnormalities associated with XP

Cutaneous Abnormalities	Neurological Abnormalities
Areas of hypo- and hyper-pigmentation	Microcephaly
Freckles	Progressive mental retardation
Xerosis (dryness) and scaling	Ataxia and choreoathetosis
Telangiectasia and atrophy	Sensineural deafness
Actinic keratoses	Spasticity
Basal and squamous cell carcinomas	
Malignant melanomas	
Ocular Abnormalities	
Photophobia	
Conjunctivitis of sun exposed area	
Entropion due to atrophy of the eyelids	
Exposure keratitis	
Neoplasms of the eyelids, conjunctiva and cornea	(Kraemer, *et al.*, 1987)

The median age of first onset of skin neoplasm is 8 years, nearly 50 years earlier than in the general population. This may, in fact, be the largest reduction in age of onset of neoplasm of any documented recessive genetic human disease. The frequency of basal cell carcinomas, squamous cell carcinomas and melanoma of the skin is 2000

times greater than in the general population for patients under 20 years of age (Kraemer, *et al.*, 1984).

Along with their increased susceptibility to cancers induced from exposure to the sun XP patients are also 10 to 20 times more likely to develop internal neoplasms (Kraemer, *et al.*, 1984), as a consequence of defects in DNA excision repair. Cultured fibroblasts from XP patients are sensitive to the types of chemical carcinogens that induce bulky DNA adducts, which would normally be repaired by the excision repair pathway (Protic-Sabljic, *et al.*, 1985). A cell line derived from a XP patient was used in the investigation of the effect of α-acetoxytamoxifen induced DNA adducts (Chapter 4).

1.6 Detection of DNA base damage

There are a variety of techniques used to detect DNA base damage. These range from methods which rely on the ability of antibodies to bind to the DNA lesion, such as the immunoslot blot technique, through to direct detection of DNA adducts using high performance liquid chromatography (HPLC), or ^{32}P-postlabelling coupled to a thin layer chromatography (TLC) or HPLC separation step. The immunoslot blot technique has been used to good effect in the detection of malondialdehyde DNA adducts in human white blood cell and gastric biopsies (Leuratti, *et al.*, 1998). Many of these assays can typically detect DNA adducts to as low as one or two per 10^9 nucleotides of DNA. However, when humans are exposed to environmental or therapeutic doses of carcinogens or drugs, the levels of DNA adducts formed can be an order of magnitude lower than this. To detect these low levels of adducts a new

method was developed at the AMS facility at Lawrence Livermore National Laboratory, which can measure very low levels of ^{14}C-labelled carcinogen bound to DNA or protein. AMS has been used to detect DNA adducts at levels of 1-10 adducts per 10^{12} nucleotides, which is less than 1 modification per cell, following acute and chronic exposure to ^{14}C-labelled carcinogens (Turteltaub, *et al.*, 1993). The ^{32}P-Postlabelling assay has been used to detect tamoxifen-DNA and benzo(*a*)pyrene diol epoxide-DNA adducts in this thesis and is therefore described in detail below.

1.6.1 ^{32}P-Postlabelling

The ^{32}P-postlabelling technique (Figure 1.7) is a very sensitive assay developed by K. Randerath and co-workers (Randerath, *et al.*, 1981) and can detect DNA damage induced by a wide range of genotoxic chemicals. The standard technique involves enzymatic hydrolysis of DNA to deoxyribonucleoside 3'-monophosphates using micrococcal nuclease (MN) and calf spleen phosphodiesterase (CSPD). MN cleaves DNA to yield strand breaks with a 5'-OH terminus to which CSPD mediates 5'-3' exonucleolytic digestion. The enzyme T4 polynucleotide kinase is then used to transfer ^{32}P- from [γ^{32}P] ATP to the deoxyribonucleoside 3'-monophosphates to yield deoxyribonucleotide 3'-5'-bisphosphates. The radiolabelled nucleotides are then applied to the origin of a polyethyleneimine (PEI)-cellulose TLC plate and separated in two directions using high molarity urea solvents. The DNA adducts are then visualized as a pattern of spots following autoradiography, typically at-70 °C (Figure 1.8).

^{32}P-Postlabelling Assay

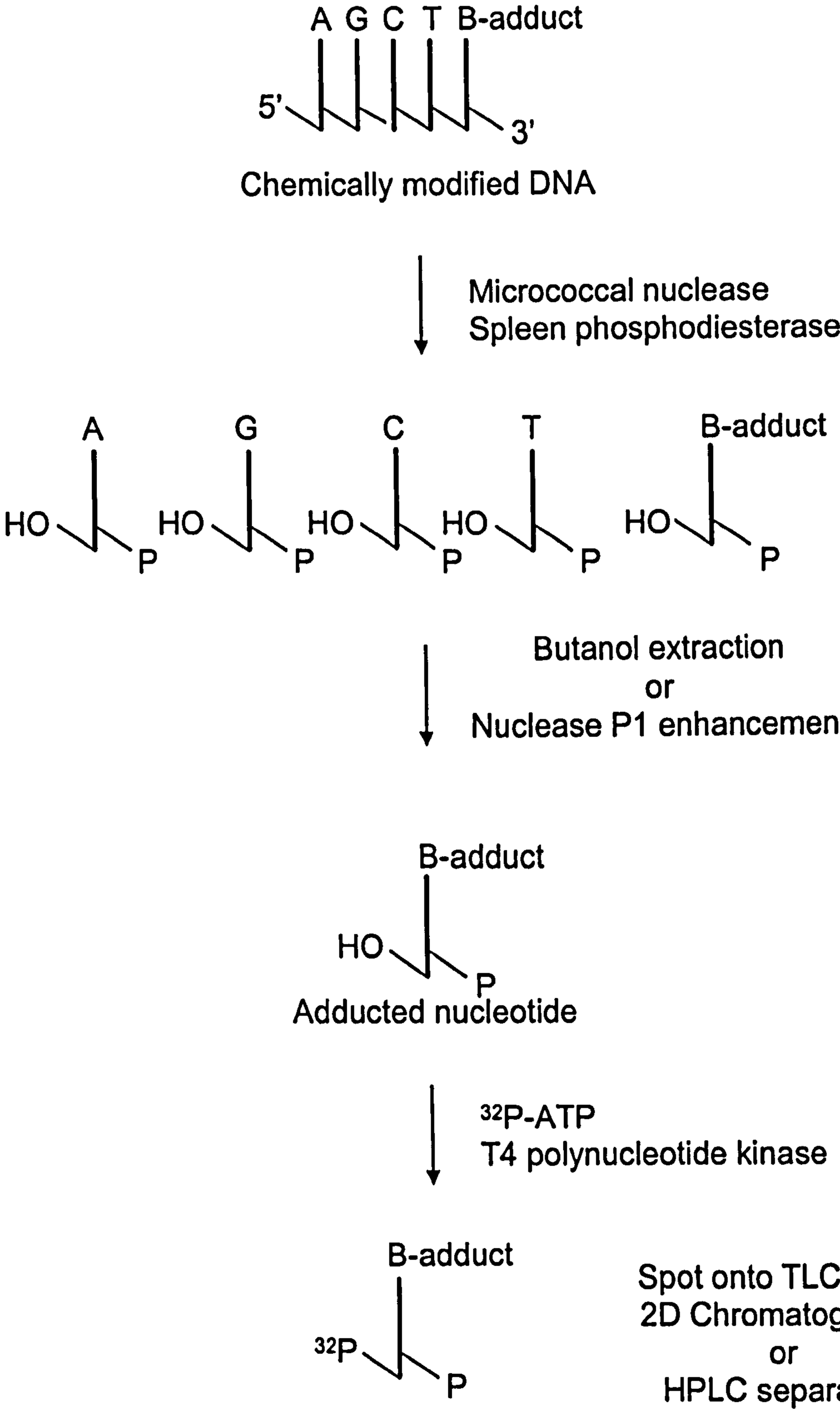


Figure 1.7 Outline of ^{32}P-postlabelling assay.

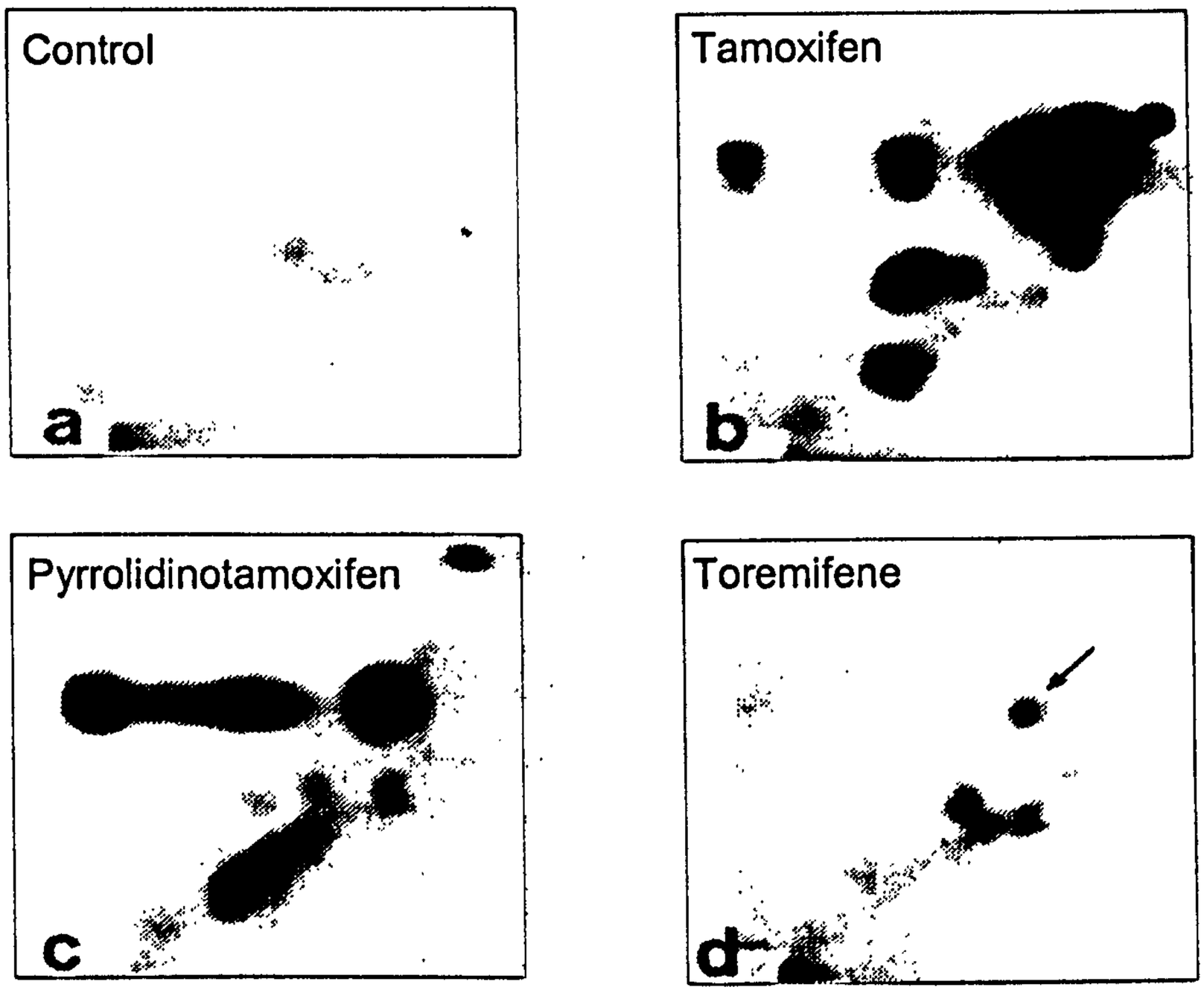


Figure 1.8 An example of radioactive TLC plates separating DNA adducts formed by control (a), tamoxifen (b), pyrrolidinotamoxifen (c), and toremifene (d) treatments. Adapted from White, *et al.*, 1992.

The radioactivity in each of these spots can be quantified by excising the area from the plates followed by scintillation counting or more recently using phosphorimagers. The amount of radioactivity in the unadducted nucleotides is obtained in a similar way using solvents which separate the ^{32}P-labelled nucleotides in one direction only. The relative adduct labelling (RAL) can be calculated according to Gupta (1985):

$$RAL = \frac{\text{dpm in adduct(s)}}{\text{dpm in normal nucleotides} \times \text{dilution factor}}$$

Adduct levels can be calculated by assuming that 1μg DNA equals 3.24×10^9 attomole nucleotides.

The standard postlabelling assay has the sensitivity to detect one adduct per 10^7 - 10^8 normal nucleotides (Gupta, *et al.*, 1982). There have been various enhancement procedures used to increase the sensitivity of the technique. These include utilisation of the enzyme nuclease P1 which modifies the normal nucleotides in such a way that they are no longer substrates for the T4 polynucleotide kinase and are therefore not labelled (3'-phosphate group is cleaved) (Reddy and Randerath, 1986). Most aromatic adducted nucleotides are resistant to the action of nuclease P1 as a consequence of steric hindrance and can therefore be ^{32}P-labelled by T4 polynucleotide kinase. An alternative enrichment step, using butanol extraction in the presence of the phase transfer agent tetrabutyl ammonium chloride, concentrates the adducts by virtue of their increased hydrophobicity. Normal nucleotides are removed by back extraction with water (Gupta, 1985). HPLC (Dunn and San, 1988) and immunoaffinity techniques (Cooper, *et al.*, 1992) have also been utilised to isolate adducts prior to labelling and remove the unadducted nucleotides. Using one of these enhancement procedures can increase the sensitivity of the technique so that one adduct per 10^{10} normal nucleotides can be detected (Phillips, 1997). The use of HPLC coupled with online radiochemical detection instead of TLC (Martin, *et al.*, 1998) has considerably increased the reproducibility of the ^{32}P-postlabelling assay (For example, Figure 1.9).

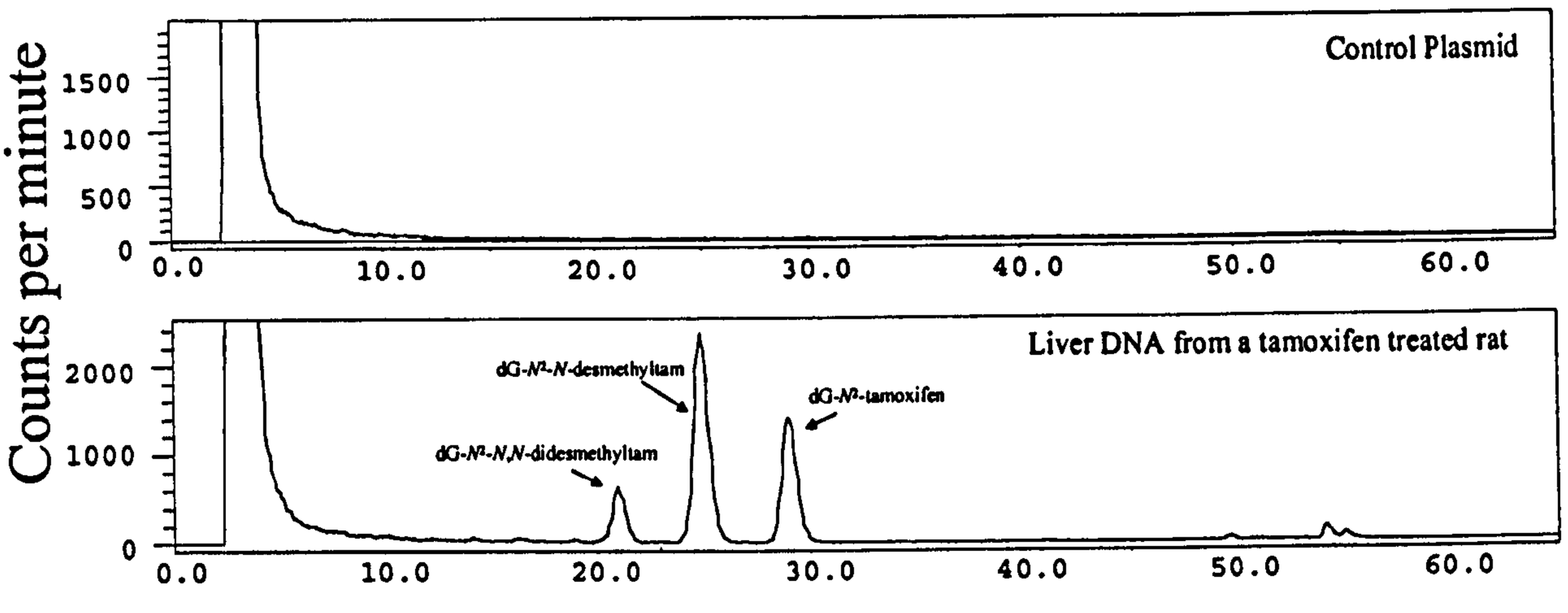


Figure 1.9 An example of radioactive HPLC chromatograms separating DNA adducts formed in Wistar Han rats by tamoxifen treatment (40 mg/kg tamoxifen in diet for 6 months).

Once DNA adducts have been detected, using a method such as ^{32}P-postlabelling, characterisation of the DNA lesion can be determined using techniques such as mass spectrometry or nuclear magnetic resonance (NMR) if sufficient quantities can be prepared. When the amount of available DNA adduct is too small then synthetic adducts have to be used; using, for example, *in vitro* treatment of calf thymus DNA with the mutagen or its metabolites. These techniques have been used to good effect in the structured elucidation of DNA adducts derived from the breast cancer drug tamoxifen (Marques and Beland, 1997; Rajaniemi, *et al.*, 1999).

1.6.2 Mass spectrometry

A mass spectrometer is a device that allows the separation of ions according to their masses. Mass spectrometers have the following elements: Firstly, an inlet through which samples can be introduced. This can be via an injection valve such as a

Rheodyne injector, a syringe pump, or a direct insertion probe. Alternatively, the outlet from a HPLC or gas chromatography (GC) system can be coupled to the mass spectrometer and samples are introduced via capillary tubing. The second element of a mass spectrometer is the source, which is responsible for the ionisation of compounds, generating charged species. There are many different forms of ionisation techniques currently in use (reviewed in: Roboz, 2002), including electrospray (ESI) (Dole, *et al.*, 1968), fast atom bombardment (FAB) (Barber, *et al.*, 1981), atmospheric pressure chemical ionisation (APCI) (Bruins, 1991), electron ionisation (EI) (Busch, 1995), chemical ionisation (CI) (Busch, 1996), matrix assisted laser desorption (MALDI) (Karas and Hillenkamp, 1988), thermospray and plasma desorption. ESI, FAB and APCI ionisation are considered to be less destructive ionisation techniques (consequently, referred to as 'soft' ionisation techniques) and thus they are considered to be more applicable to applications such as characterisation of DNA adducts and drug metabolites.

The next element of a mass spectrometer is one or a number of analysers which are responsible for the separation of charged species according to their mass to charge ratio. Sector instruments incorporate an electro-magnetic field while quadrupole mass spectrometers incorporate a combination of radio-frequency and direct current fields. Further analysers are used if characterisation of specific molecules is required. Ions can be selected from the first analyser and repelled into a collision cell, where they are fragmented using a neutral gas such as helium, argon or xenon. Product ions are then selected and repelled towards a second analyser, where the individual product ions are separated. The ions are then sequentially focused at a single point: the detector.

1.6.2.1 Electrospray ionisation.

An electrospray is produced by applying a strong electric field, under atmospheric pressure, to a liquid passing through a capillary tube with a low flow rate (Figure 1.10). The electric field is obtained by applying a potential difference of 3-6 kV between the capillary and the counter electrode, separated by 0.3-2.0 cm. This field induces a charge accumulation at the liquid surface located at the end of the capillary, which will break to form highly charged droplets.

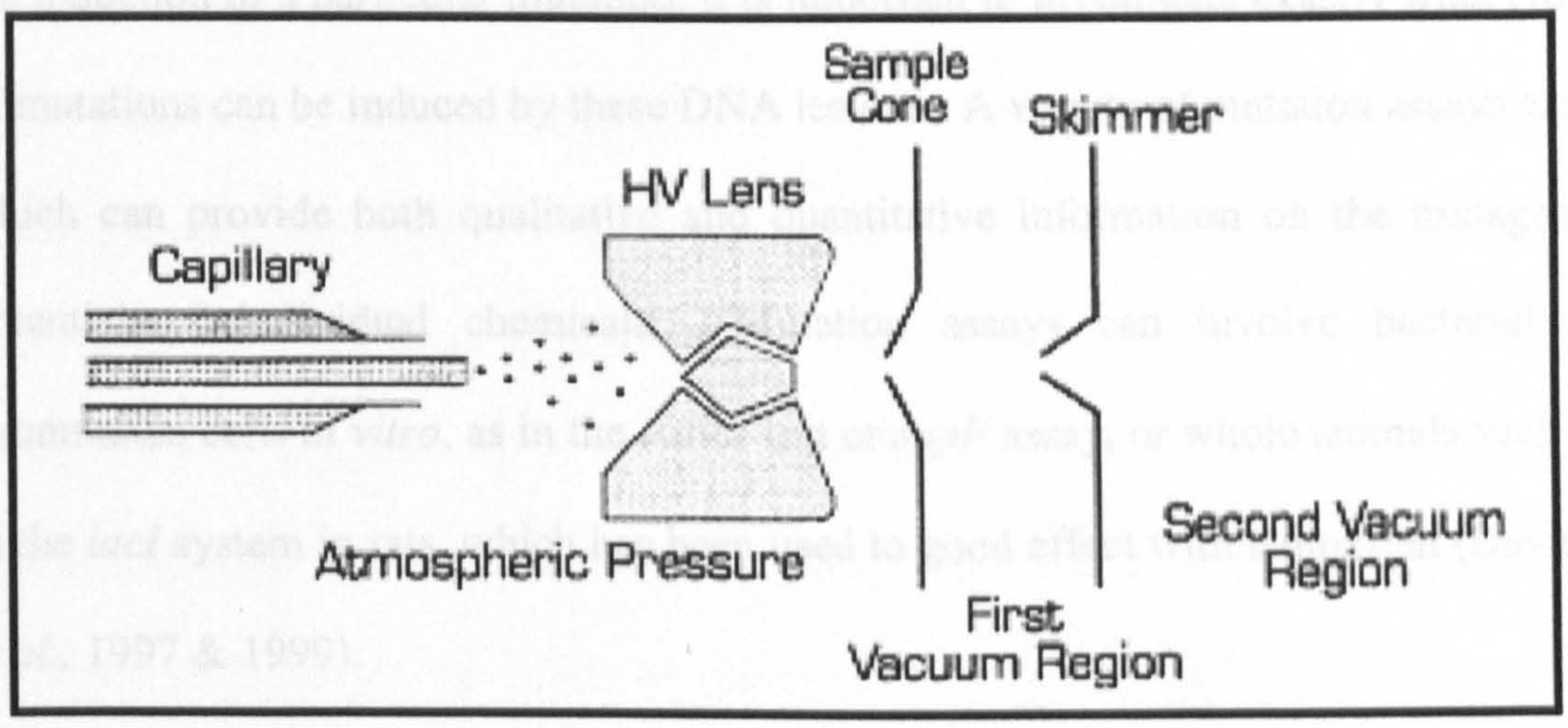


Figure 1.10 The typical configuration of an electrospray interface. (Adapted from Gaskell, 1997).

The electric field and the sheath of gas combine to create a mist of highly charged droplets, which pass down a potential and pressure gradient towards the analyser portion of the mass spectrometer. During this passage, the solvent contained within the droplets evaporates, causing them to shrink to the point where the repelling coulombic forces come close to their cohesion forces, thereby resulting in their explosion. The droplets then undergo a cascade of ruptures, yielding smaller and smaller droplets until the electric field on their surface becomes large enough to produce the desorption of the ions (Gaskell, 1997). The ions that are obtained carry a

great number of charges if several ionisable sites are present on the molecule (Gaskell, 1997).

1.7 Detection of mutations

Detecting the presence of DNA adducts on different DNA bases is not enough to elucidate any deleterious biological effects due to the chemical. In order to understand the mechanisms involved in mutagenesis and the factors responsible for the induction of a particular mutation, it is important to investigate exactly what types of mutations can be induced by these DNA lesions. A variety of mutation assays exist which can provide both qualitative and quantitative information on the mutagenic potential of individual chemicals. Mutation assays can involve bacterial or mammalian cells *in vitro*, as in the Ames test or *supF* assay, or whole animals such as in the *lacI* system in rats, which has been used to good effect with tamoxifen (Davies, *et al.*, 1997 & 1999).

1.7.1 The Ames test

Directly assaying potential carcinogens by testing for their ability to form tumours in animals is difficult and expensive. However, as well as causing tumours in animal cells, most carcinogens are also mutagens. Bruce Ames and colleagues (1973) developed a simple, indirect assay for identifying potential carcinogens, based on this principle. The assay is based upon the reversion of mutations in the histidine (*his*) operon in the bacterium *Salmonella typhimurium*. The *his* operon codes for enzymes which are required for the synthesis of the amino acid histidine. Strains which have mutations in the *his* operon cannot produce histidine and so are unable to grow on histidine lacking media. These are known as histidine auxotrophs. Mutations induced

in the *his* operon, when bacteria is treated with a test mutagen, will result in restoration of the His^+ phenotype and bacteria are then able to grow on minimal plates lacking histidine (revertants). This provides a simple, sensitive method of screening for mutagens. This is known as a back mutation assay, since the mutagen is allowing the gene to regain its function, or go back to normal. Mutation assays such as the *supF* assay, are known as forward mutation assays, resulting in a null mutation as the gene function is removed.

Many chemicals are only carcinogenic (or mutagenic) after *in vivo* metabolism. To investigate whether chemicals require metabolic activation they are tested with and without the addition of an S9 extract from rat liver. To produce the metabolizing system rats are injected with Aroclor (a polychlorinated biphenyl mixture) to induce the expression of the various enzymes involved in metabolic activation of chemicals. The livers of these rats are then homogenised and centrifuged and the supernatant (S9 mix) added to the bacterial plates. The assay is comprised of the following steps (Ames, *et al.*, 1973). Several different *S.typhimurium* strains, along with the agents to be tested, are plated on minimal medium plates containing a limiting amount of histidine. This amount of histidine allows the bacteria to undergo several generations of growth, allowing the procession of DNA lesions into mutations and therefore for the expression of the mutant phenotype. After the histidine has been exhausted, His^+ revertants will grow as distinct colonies, each one representing an independent mutational event. Positive (containing known mutagens) and negative controls are used along with repeat plates containing S9 mix. Since there is enough histidine for a small amount of growth, negative control plates will have a lawn of bacterial growth. Any mutant colonies will be seen to grow on top of this lawn as larger distinct

colonies. It is the relative number of colonies seen compared to both the positive and negative controls which gauges the mutagenicity of the test compound.

Several strains of *S.typhimurium* possessing different mutations in the *his* operon are used to test for different classes of mutagens. For example; mutagens that induce base substitutions compared to those that induce frameshift mutations (insertions or deletions). Along with the ability to select for different mutations, the *S.typhimurium* used have three more properties that make them more sensitive to mutagens (Mortelmans and Zeiger, 2000). These are, i) a mutation in the *rfa* gene which makes the outer membrane more permeable to large molecules, ii) the deletion of the *uvrB* gene, to eliminate excision repair of DNA damage, and iii) the possession of pKM101 plasmid which increases error-prone repair of DNA damage. These attributes allow for a very sensitive test for a broad range of mutagens. For this reason the Ames test has become one of the standard *in vitro* tools for the development and testing of new pharmaceuticals.

The Ames test is a reversion assay, and so only the specific mutation needed to restore the mutation in the *His* gene to wild type can be detected for each *His*$^-$ strain of *S. Typhimurium* used. A number of forward mutation assays exist that allow for the generation of mutations at most sites within the target gene. A commonly used gene codes for hypoxanthine-guanine phosphoribosyl transferase (*hprt*) (Albertini, *et al.*, 1982), in which mutations are readily detected by altered sensitivity of the cells to anti-proliferative drugs, such as 8-azaguanine. This is incorporated into DNA and stops DNA synthesis. The enzyme is necessary to convert the drug into the nucleoside monophosphate, essential for its incorporation into DNA. If mutant (inactive) enzyme is present, 8-azaguanine is not incorporated into DNA and the cells

proliferate whereas non-mutant cells are killed. For example, one assay uses the human gene for hypoxanthine-guanine phosphoribosyl transferase in the T-cell cloning assay (Hou, *et al.*, 1995). This assay can provide insights as to whether mutations in the marker gene (*hprt*) in surrogate cells (i.e. lymphocytes) can predict health consequences such as carcinogenesis.

1.7.2 SupF assay

Another forward mutation assay, the *supF* assay, uses the *supF* tRNA gene as the target for mutation. This assay has been used throughout this project for the detection of mutations induced by reactive metabolites of tamoxifen, BPDE and UV light. In the *supF* assay, developed by Seidman and co-workers (1985), the *supF* target gene is located on a shuttle vector plasmid that can replicate in both primate and *Escherichia coli* cells. The plasmid is treated with a carcinogen and then transfected into mammalian cells where DNA repair and/or mutagenesis can take place. Plasmid DNA is removed and transformed into prokaryotic cells to screen for mutant colonies, which indicate whether DNA mutations are present (Figure 1.11).

The *supF* gene encodes an amber suppressor tRNA (Kraemer and Seidman, 1989). An amber mutation is where a codon in the DNA has been mutated to the CTA stop codon. The mRNA that is transcribed from this gene will contain the UAG (amber) stop codon that is the signal for translation of the message to terminate. The presence of the stop codon within a coding sequence will therefore lead to the production of a truncated, inactive, protein. The wild-type *supF* gene is itself a mutant tyrosine tRNA gene, containing the CUA anticodon, which inserts tyrosine at the amber stop codon (UAG). Hence, the *supF* tRNA can suppress the amber mutation and allow

production of a full length, active protein. The *supF* gene is therefore a non-essential sequence which has been extensively used in a number of shuttle-vectors to look at mutagenesis from a wide range of carcinogenic compounds (for example; Maher, *et al.,* 1989; Levy, *et al.,* 1992; Routledge, *et al.,* 1994).

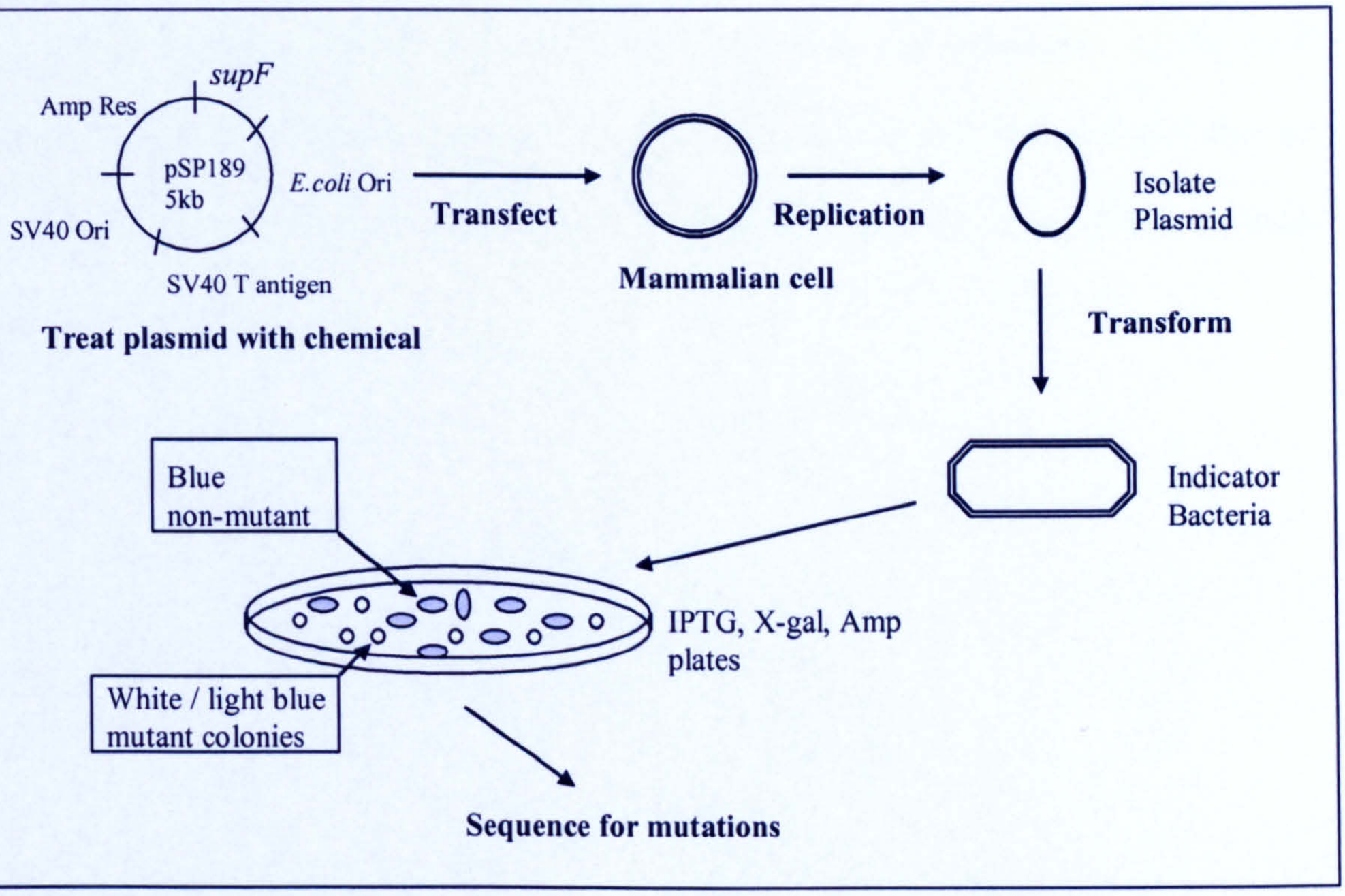


Figure 1.11 Outline of the *supF* assay method.

The advantage of the *supF* system is that a wide variety of changes can be induced and detected; all six possible base substitutions, insertions and deletions. The tRNA sequence itself or its promoter can be mutated. This means that the *supF* gene gives a very sensitive measure of mutations (Seidman, *et al.,* 1985). It is also possible to analyse DNA damage in the same DNA used for the induction of mutations.

In this system the amber mutation is in the *lac*Z gene of the *lac* (Lactose) operon in the indicator strain of *E. coli* (MBM7070). The *lac* operon is a grouping of genes coding for proteins used in the breakdown of lactose to β-galactose and glucose. The *lac*Z gene codes for β-galactosidase, which is the enzyme responsible for cleaving the disaccharide lactose to the monosaccharide units of glucose and galactose. Therefore this strain of *E. coli* cannot convert lactose to glucose and galactose. In the *supF* assay (Figure 1.12) the bacteria that have been transformed with the *supF* plasmid are grown on plates containing the lactose analogue, 5-bromo-4-chloro-3-indolyl-β-D-galactose (X-gal).

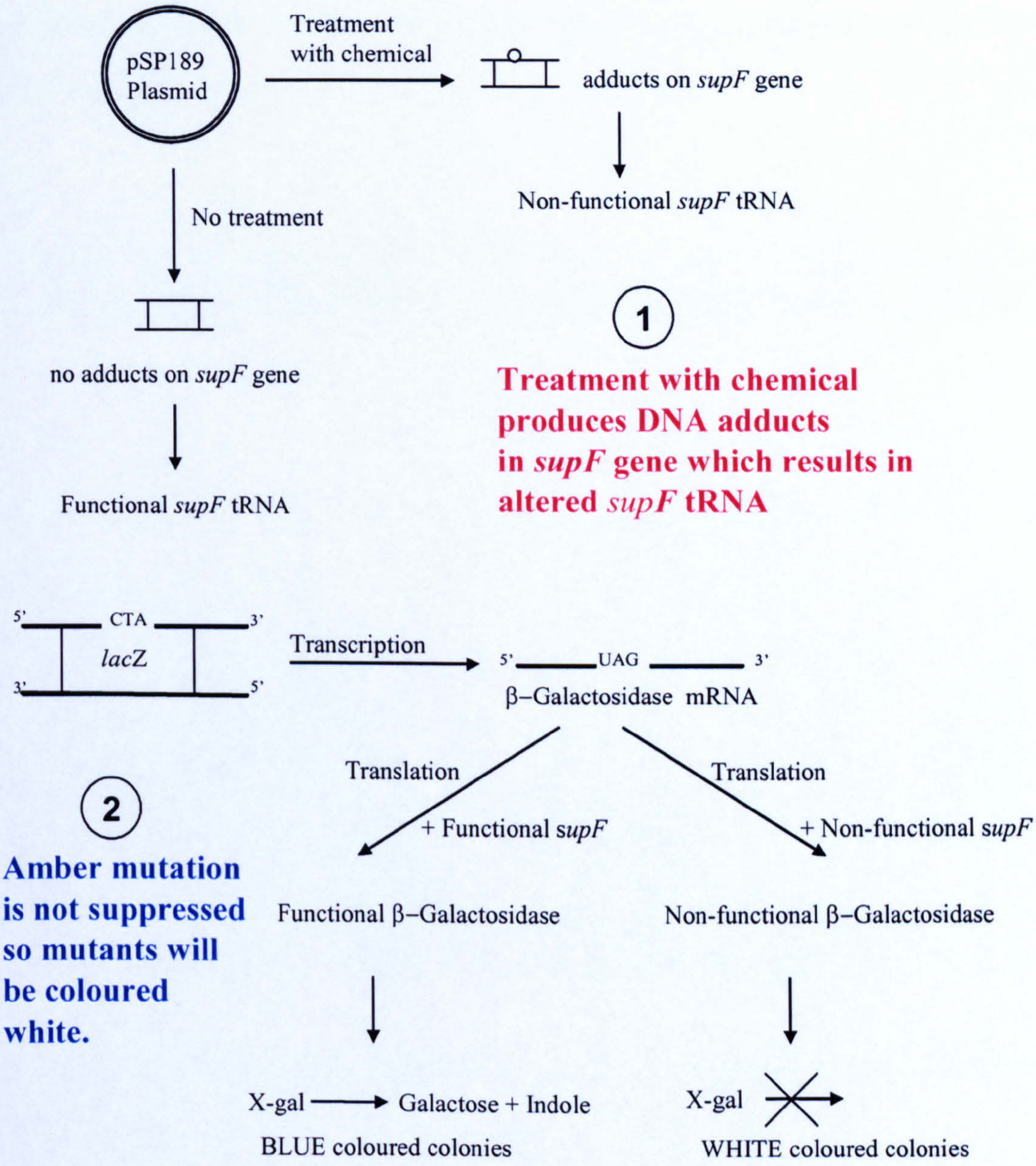


Figure 1.12 Flow diagram of *supF* assay.

X-gal yields a blue product when cleaved by β-galactosidase. If the *E. coli* contain the wild-type *supF* gene, they will be able to produce active β-galactosidase, and will form blue colonies. White colonies will, therefore, contain mutated *supF* gene. When mutated *supF* gene DNA is sequenced from a number of white colonies, a spectrum of mutations can be obtained, which show the types of mutations induced by the mutagenic agent, and the distribution of these mutations in the *supF* gene. Figure 1.13 illustrates a sequencing electropherogram from a pSP189 plasmid containing a

non-mutant *supF* gene, which was extracted from a blue colony grown on an X-gal containing plate.

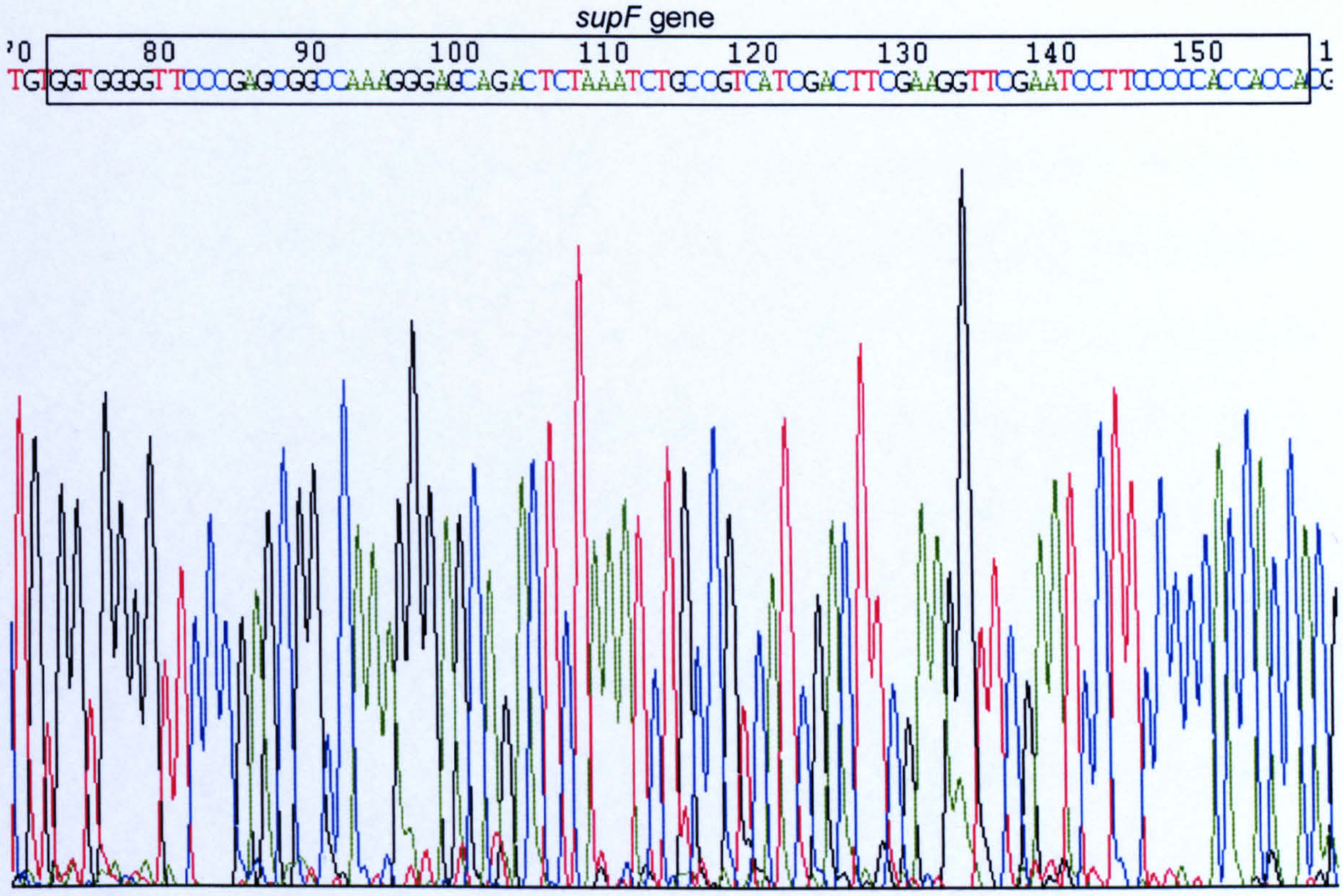


Figure 1.13 Electropherogram illustrating a sequence containing the *supF* gene from pSP189 plasmid. No mutations are present.

The original assay has been further developed by the addition of an 8 base 'signature sequence' giving 4^8 (65,536) possible unique sequences (note that this figure differs from that of 2 x 4^8 given in the original reference (Parris and Seidman, 1992), as insertion of the sequence in either direction does not double the number of possible sequences) . This means that plasmid with mutations at identical positions can be identified as 'siblings', those arising from the same initial event, or arising from independent events. Previously, identical mutations were not included if they

appeared on the same transfection plate. Identical mutations can now be included in mutation spectra if they have different signature sequences.

1.8 Aim of Ph.D. project

The aim of the work described in this thesis was to validate the *supF* assay in our laboratory by using UVC radiation to introduce damage into the pSP189 plasmid shuttle vector. Once validated the *supF* assay would then be used to investigate the mutagenicity of reactive metabolites of tamoxifen, α-acetoxytamoxifen and 4-hydroxytamoxifen quinone methide (4-OHtamQM), in a variety of cell lines. Cells both deficient and proficient in nucleotide excision repair were used to investigate the effect of DNA repair on α-acetoxytamoxifen induced DNA damage. The mutagenicity of BPDE and UV radiation was investigated using binary treatments. The mutagenicity of tamoxifen-DNA adducts was to be investigated using a site-selective mutation assay. The site-selective mutation assay was developed using the *supF* assay and mass spectrometric analysis to detect mutations in synthetic oligonucleotides.

Chapter 2

Mutations induced in the pSP189 *supF* gene by exposure to 254 nm ultraviolet (UVC) radiation

2.1 Introduction

Although it is agreed that chemical mutagens and carcinogens are responsible for the onset of the majority of cancers, non-chemical carcinogens such as ultraviolet (UV) light also have their role to play in cancer initiation. Ultraviolet light is continuously bathing our environment. It is not just when on holiday in the sun that we are at risk but even at home or in the office we still become exposed to UV radiation. Both fluorescent and tungsten-halogen lighting emit significant amounts of UV radiation. Since the recent scares on the depletion of the ozone layer we can assume that our exposure levels to UV irradiation will also increase.

Ultraviolet radiation consists of electromagnetic waves of the wavelength 100 to 400 nm compared to visible light in the range of 400 to 700 nm. UV radiation is generally sub-divided into three regions: UVA, 320 to 400 nm; UVB, 280 to 320 nm; and UVC, 100 to 280 nm (Figure 2.1). UVB radiation exposure has major health implications because it induces the symptoms of sunburn and adaptive responses to exposure such as hyperpigmentation and skin thickening. The shorter wavelength UVC radiation is mainly absorbed by the stratosphere, by reacting with ozone, or clothing and layers of dead skin so gives us little cause for concern over health issues. The biological role of UVA radiation is not fully understood. Exposure levels to UVA do not vary with time of day or season which may suggest a lack of association with skin cancer induction although there are indications that it can affect tissue injury in conjunction with UVB and certain photosensitizing chemicals (Urbach, 1993).

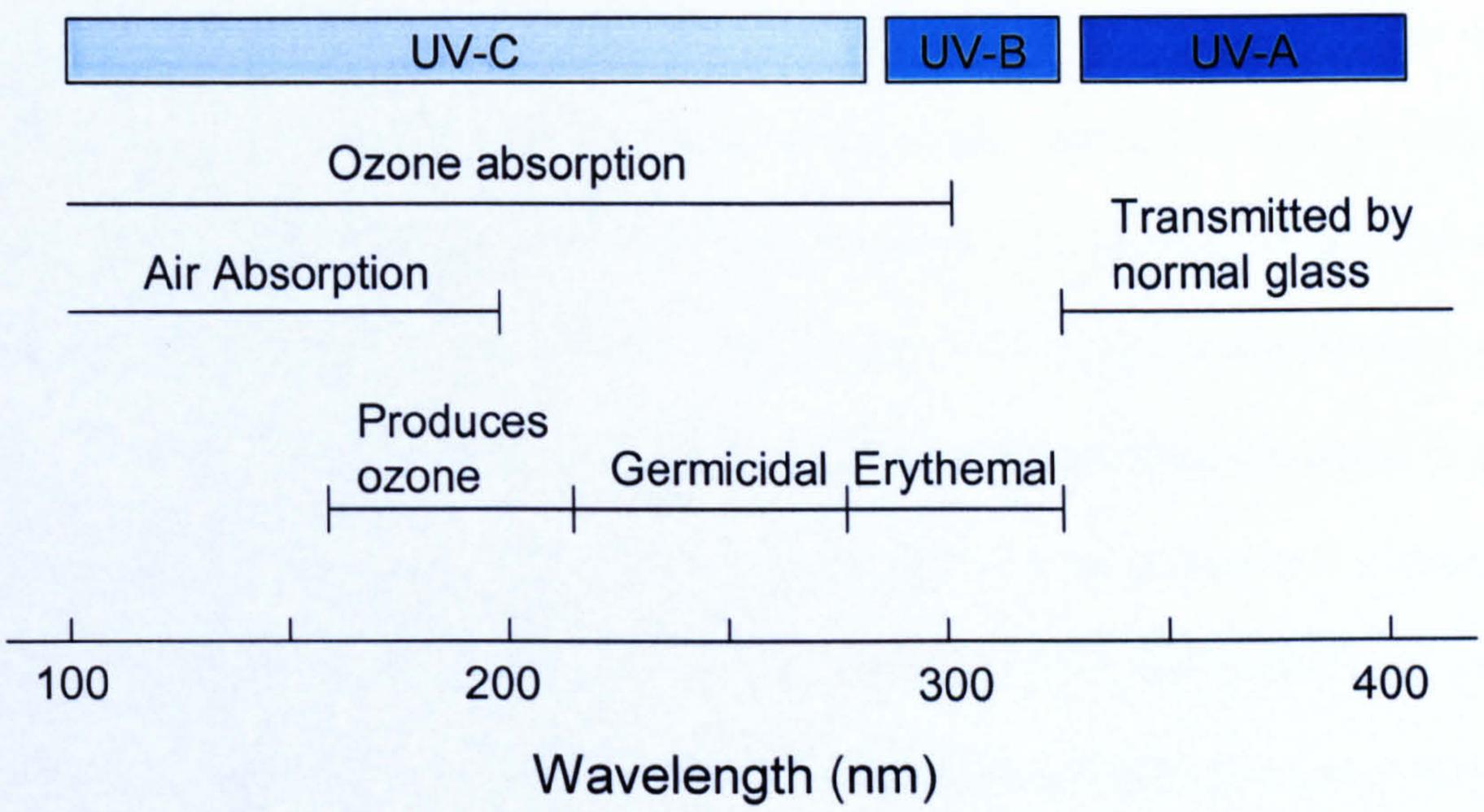


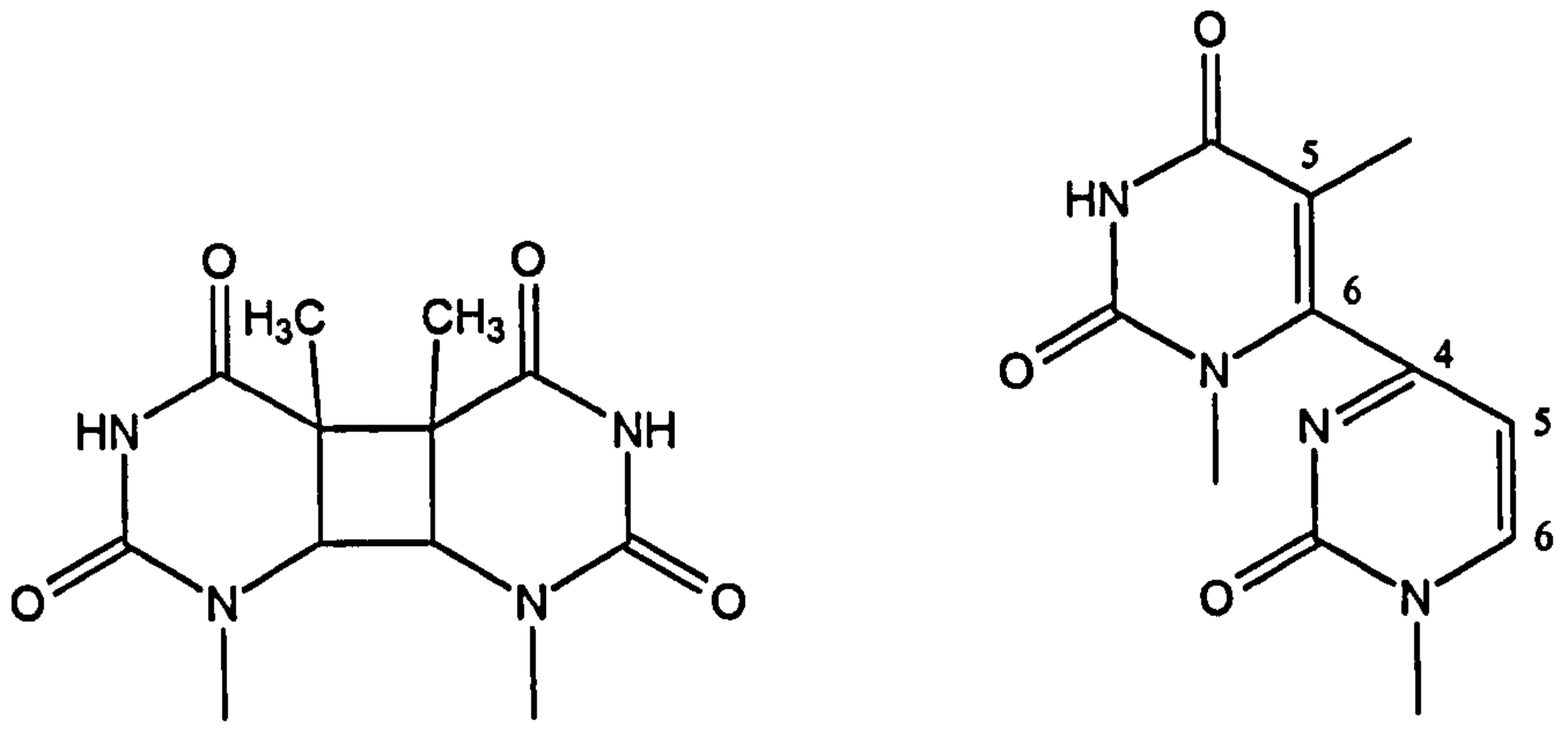
Figure 2.1 UV radiation spectrum

Circumstantial experimental and epidemiological evidence has implicated UV radiation in the induction of both basal cell and squamous cell carcinomas, the most common and generally the most easily treated forms of cancer (Urbach, 1993: Marks, 1996). Recent generations have found their leisure time to have increased leading to an increase in outdoor pursuits, and with this an increase in skin cancer incidence. Epidemiological studies have shown that tumour incidence correlates positively with circumstances that elevate cumulative skin exposure to UV radiation (Vitaliano, 1978). Thus, for instance, tumour incidence increases with decreasing latitude (Elwood, *et al.,* 1974). Furthermore, tumours arise predominantly in individuals and ethnic groups with weakly pigmented skin (Chuang, *et al.*, 1990). Tumours also tend to appear on body surfaces which receive the greatest exposure, such as the head and neck (Haenszel, 1963). People in occupations that require greater outdoor exposure clearly have a higher tumour incidence; e.g. farming, police work, etc. (Vitaliano, 1978).

Malignant melanoma, the highly aggressive cutaneous cancer of melanocytes, is of growing concern since it primarily affects young adults. Its incidence is increasing faster than any non-cutaneous cancer and is often lethal. Melanoma incidence is also associated with the sun (IARC, 1992). This relationship is more complex than with other skin cancers since it seems to involve intense but intermittent exposures to sun. For example, melanoma incidence in individuals with outdoor occupations is actually lower than for those receiving intermittent exposures (Lee and Strickland, 1980; Garland, *et al.*, 1990). Melanoma incidence also increases with decreasing latitude (Armstrong, 1984) and is predominantly seen in white-skinned populations (Muir, *et al.*, 1987), as do other skin cancers. Other factors for risk include the extent of intermittent exposure to the sun, susceptibility to sunburn and the numbers of dysplastic nevi, possible precursors to melanoma, (Armstrong, 1988) although the actual frequency of sunburn shows no correlation.

2.1.1 UV induced DNA Damage

UV radiation induces the formation of pyrimidine cyclobutane dimers (Beukers and Berends, 1960) and pyrimidine-pyrimidone (6-4') photoproducts (Lippke, *et al.*, 1981), both of which are formed between adjacent pyrimidine bases (Figure 2.2). Traditionally, cyclobutane dimers have been considered as bulky, helix-distorting lesions which are strictly noncoding and whose presence results in the arrest of replication as no base can be inserted which can form stable hydrogen bonds with the lesion (Witkin, 1976, Chan, *et al.*, 1985).



Cyclobutane Dimer 6-4'Photoproduct

Figure 2.2 UV light catalyses the formation of pyrimidine cyclobutane dimers and 6-4' photo-products between adjacent pyrimidines.

More recent work, using duplex decamers, has shown that the major T-T dimer can be accommodated inside the DNA double helix with little distortion (Taylor, *et al.*, 1990). This has shown that correct hydrogen bonding with adenine is still possible, also supported by *in vitro* DNA synthesis experiments (Taylor and O'Day, 1990; O'Day, *et al.*, 1992). The yield of cyclobutane pyrimidine dimers is influenced by the nucleotide composition of the DNA. It has been found that the yield of C-C photoproducts is significantly lower than that of C-T, T-C or T-T (Mitchell, *et al*, 1992) when a plasmid was treated with UV radiation. Investigations looking at the sequence context of cyclobutane pyrimidine dimers, in a fragment of the *E.coli lacI* gene, showed that there was a preference for dimer production at TT sites which were flanked on both sides by A, rather than by A (5' side) and G (3' side) (Gordon and Haseltine, 1982). This nucleotide-flanking effect could not account for all variations seen, as the frequency of dimerisation was further altered by the sequence surrounding the ATTG sites. This study also showed that the secondary structure of the DNA did not effect dimer production, although differences in the overall rate of pyrimidine

dimer formation in single and double-stranded DNA have been detected in other studies (Setlow, 1966, & 1968, Varghese, 1972). These experiments demonstrate a 'non-random' formation of cyclobutane dimers, although sites of formation and dimer yields cannot be extrapolated between doses and experiments.

Alkali-labile lesions at positions of cytosine (and, much less frequently, thymine) located 3' to pyrimidine nucleosides are also seen in UV irradiated DNA (Lippke, *et al.*, 1981). These are known as pyrimidine-pyrimidone (or 6-4') photoproducts. The 6-4' products of TC, CC, and to a lesser extent, TT sequences are all observed in DNA irradiated with UV, whereas that of CT is not. These 6-4' photoproducts introduce a major distortion into the double helical structure of DNA (Taylor, *et al.*, 1988). At most sites in DNA 6-4' photoproducts occur at a frequency several fold lower than that of pyrimidine dimers (Brash and Haseltine, 1982). At some sites, though, these lesions were detected at levels equal to or greater than that of dimers.

Other photochemical reactions can be catalysed by UV irradiation, these include the spore photoproduct (5'-thyminyl-5,6-dihydrothymine) produced in spores of *Bacillus subtilis* (Varghese, 1970), involving adenine (Bourre, *et al.*, 1987), cytosine hydrate, where a water molecule is added across the 5,6 double bond (Fisher and Johns, 1976); and thymine glycols also resulting from saturation of the 5,6 double bond (Demple and Linn, 1982). UV radiation can result in the cross-linking of DNA to proteins (Peak and Peak, 1986) or to other DNA duplexes (Marmur and Grossman, 1961). Irradiation of DNA with UV radiation has also been shown to result in the breakage of the polynucleotide chain (Rosenstein and Ducore, 1983, Peak and Peak, 1986, Routledge, *et al.*, 2001) although at very high doses. Irradiation at longer

wavelengths dramatically increases the frequency of both strand breaks and DNA-protein crosslinks (Tyrrell, 1991).

The involvement of dimer formation in carcinogenesis is strongly supported by studies of the genetic defect *Xeroderma pigmentosum (XP)*, a complex of disorders characterised by deficient excision repair of UV-induced pyrimidine dimers and a high incidence of skin cancer (Kraemer, *et al.*, 1987, also see chapter 1). *In vitro* studies have shown that the UV action spectrum for transformation of hamster embryo cells (Doniger, *et al.*, 1981) or human embryonic fibroblasts (Sutherland, *et al.*, 1981) is consistent with that for UV induced dimer formation.

2.1.2 Mutagenesis of UV radiation

Living cells have developed a range of mechanisms which can be used for the removal of UV induced pyrimidine photo-products. These range from the photo-reaction of DNA in both prokaryotes and eukaryotes (Dulbecco, 1949, Kelner, 1949, Sancar, 1990) to the removal of the appropriate lesion using nucleotide excision repair (Chapter 1). If for some reason these lesions are overlooked then at the next round of replication, mutations can be introduced into the DNA molecule. Many studies have investigated the mutability of DNA after UV irradiation. There exists a correlation between the frequency of UV-induced damage at TC and CC sequences in the *E.coli lacI* gene and the frequency of nonsense mutations. This correlation is better for 6-4' photoproducts than for pyrimidine dimers (Brash and Haseltine, 1982, Franklin and Haseltine, 1986). Irradiation of 6-4' products with 313 nm light (UVB) leads to the production of the Dewar isomer (Taylor and Cohrs, 1987), a lesion which may have significant biological relevance, although the T-T derived isomer exhibits mutagenic

behaviour in accordance with the 'A rule' (Lee, *et al.*, 2000). The 6-4' photoproduct has been found to be much more mutagenic (LeClerc, *et al.*, 1991) than the Dewar isomer. When a single stranded vector (containing the *E.coli supF* gene) was transfected into monkey (COS7) cells, UV irradiation (254 nm, UVC) was found to preferentially induce GC→AT transitions (Madzak, *et al.*, 1992). Treatment with UVC radiation preferentially induces GC→AT transitions in human *Xeroderma pigmentosum* derived cells using a double stranded vector containing the *E.coli supF* gene (Bredberg, *et al.*, 1986). UVB radiation (313 nm) also induces GC→AT transitions in the *supF* gene when irradiated plasmid was transfected into African green monkey kidney cells (CV-1) (Keyse, *et al.*, 1988). Other systems have also suggested the GC→AT transition to be the major mutation induced by UV radiation (For example; Andrew, *et al.*, 1999; Asahina, *et al.*, 1999).

2.1.3 Aim

The aim of the work described here was to validate the *supF* assay (Seidman, *et al.*, 1985) in our laboratory by using UVC radiation (at a wavelength of 254 nm) to introduce damage into the pSP189 plasmid shuttle vector (Parris and Seidman, 1992). Many previous *supF* experiments have been carried out using UV radiation so a comparison can be made from results seen in setting up the assay and those already published.

2.2 Materials and Methods

2.2.1 Materials

All chemicals were from Sigma-Aldrich, Poole, Dorset, UK unless otherwise stated.

2.2.2 Shuttle vector plasmid, bacterial strain and cell lines

The plasmid pSP189 containing the *supF* gene (30) and *E.coli* strain MBM7070 {F$^-$, lacZamCA7020, lacγ1, hsd R$^-$, ara D139, Δ(araABC-leu) 7679, gal U, gal K, rps L, thi (derivative of MC1061) (Casadaban and Cohen, 1980)}were gifts from Dr. M. Seidman (National Institute of Aging, NIH, Baltimore, MD, USA). Human embryonic adenovirus-transformed kidney cells (Ad293) were cultured from cells provided by Dr. A. Dipple, National Cancer Institute, Frederick, MD, USA. Ad293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Life Technologies Ltd, Paisley, UK) at 37°C in 5% CO_2 in air.

2.2.3 Preparation of SOC medium

Tryptone (2 g), yeast extract (0.5 g), 1 M NaCl (1 ml), and 1 M KCl (0.25 ml) were dissolved in tissue culture grade water (97 ml) then autoclaved. Once cooled to room temperature 2 M Mg^{2+} stock solution (1 ml; 1 $MgCl_2.6H_20$ / 1 M $MgSO_4.7H_2O$, 0.2 μm filter sterilised) and 2 M glucose (0.2 μm filter sterilised) was added. The solution was made up to 100 ml with tissue culture grade water and filter sterilised

using 0.2 μm Sartorius filter units (Sartorius, Gothemburg, Germany). The pH was *adjusted to 7.0.*

2.2.4 Preparation of plates for bacterial screening assay

Ampicillin (800 μl, 50 mg/ml), X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactose, 600 μl, 50 mg/ml) and IPTG (isopropyl β-D-thiogalactoside, 200 μl, 50 mg/ml) were added to molten Agar (400 ml, 50°C). Plates were poured, 200 ml per 525 cm^2 square bioassay dish or 25 ml per 9 cm diameter round plate, and stored overnight in darkness to cure. Plates were then stored at 4°C until needed, when they would be warmed in a 37°C incubator prior to use to dry any excess moisture.

2.2.5 Production of E.coli *MBM7070 glycerol stocks*

Luria Bertoni broth (LB broth, 12 ml) was inoculated with a 1 ml aliquot of *E.coli* MBM7070 (15% glycerol in LB broth). The culture was incubated for approximately 4 hours in an orbital shaking incubator (Gallenkamp, Germany) at 37°C and 250 rpm. The starter culture was further used to inoculate a 500ml flask containing 125 ml LB broth. This was grown for 16 h at 37°C and 250 rpm. The next morning 36 ml of culture from each flask were combined with 36 ml of 30% sterile glycerol in LB broth. These were aliquoted and frozen at -80°C for future use.

2.2.6 Plasmid preparation

LB broth (2x12 ml) containing 100 μg/ml ampicillin was inoculated with a 1 ml aliquot of *E.coli* MBM7070 (15% glycerol in LB broth) containing the pSP189 plasmid. The culture was incubated for approximately 4 h in an orbital shaking incubator at 37°C and 250 rpm. These starter cultures were further used to inoculate four 2 l flasks each containing 500 ml LB broth (containing 100 μg/ml ampicillin). These were grown at 37°C and 250 rpm. After 16 h growth glycerol stocks were made as above and the plasmid was prepared from the culture using a Qiagen Endofree Mega plasmid preparation kit (Qiagen, Crawley, UK). Briefly, this involves alkaline cell lysis and separation of plasmid DNA on an anion exchange column, followed by ethanol precipitation of purified DNA. The yield of DNA was calculated from the optical density at 260 nm as shown below.

DNA concentration = A^{260} × Dilution × 50 (constant for dsDNA)

The yield of plasmid would generally be approximately 1 mg per 500 ml overnight culture. The plasmid DNA was stored at -80° C in 100 μg aliquots.

2.2.7 Treatment of pSP189 plasmid with UVC radiation

pSP189 plasmid was added to a 96 well microtitre plate (77.5 μg plasmid per well) and exposed to three different doses of UVC (254 nm) radiation using a hand held UV lamp (UVP, Inc., San Gabriel, Ca, USA): 6.8 W/m^2 for 15 s, 45 s and 90 s to produce doses of 100 J/m^2, 300 J/m^2 and 600 J/m^2 respectively. Control plasmid was protected from UV radiation by storage in a foil covered tube. The treated plasmid was stored at 4 °C prior to transfection into human (Ad293) cells.

2.2.8 Calcium phosphate mediated transfection of Ad293 cells with UVC treated pSP189 plasmid DNA

Ad293 cells (grown at 37°C, 5% CO_2, 90% confluent) were split 1 in 10 (approx 2×10^6 cells) and plated in 9 cm transfection plates in 10 ml Dulbecco's Minimum Eagle's medium (Life Technologies Ltd., Paisley, UK) (with glutamax 1 and pyridoxine, without glutamine and sodium pyruvate, 10% foetal calf serum) and grown for 68 h (37°C, 5% CO_2). Cells were 40-60% confluent prior to transfection. Three hours before transfection, used medium was aspirated and 10 ml fresh medium added. Transfection mixture (2× HEPES buffered saline [HBS]) was prepared on day of transfection from 20× stock solutions (stored at 4 °C).

Solution A $Na_2HPO_4.7H_20$, 0.376 g/100 ml (14 mM

Solution B D-Glucose, 2.16 g/100 ml (120 mM)

Solution C NaCl, 16 g/100 ml (2.7 M)

Solution D KCl, 0.744 g/100 ml (100 mM)

Solution E HEPES, 9.52 g/100 ml (400 mM)

1 ml of each stock solution (A-E) was added to a sterile polypropylene tube, the pH was corrected to pH 7.05, and then the solution was made up to 10 ml with sterile tissue culture grade water. HBS (2x solution, 0.5 ml), 2 M $CaCl_2$ (62 µl stored at -20°C) and pSP189 plasmid DNA (10 µg) [different UV treatments or control] was added to a 1.5 ml tube and made up to 1 ml with water before being incubated at room temperature for 30 minutes. The aliquot of precipitated DNA was carefully added drop wise to each transfection plate and swirled gently to mix. Plates were returned to

the incubator (37°C, 5% CO_2). Twenty-four hours after transfection used media was aspirated and replace with 10 ml fresh media. The plasmid was reclaimed from the Ad293 cells between 45-48 h after transfection.

2.2.9 Isolation of pSP189 plasmid from Ad293 cells

Media was aspirated from the transfection plates. Cells were scraped, using disposable plastic cell scrapers (Fisher Scientific, Loughborough, UK) into the residual volume of medium on each plate then pipeted into 1.5 ml micro centrifuge tubes. The plates were washed by scraping remaining cells into buffer P1 (300 µl, Qiagen, Crawley, UK). The Qiagen mini plasmid preparation protocol was followed for the remaining steps. Briefly, this involves alkaline cell lysis and separation of plasmid DNA on an anion exchange column, followed by ethanol precipitation of purified DNA. Syringes and fine needles were used to remove supernatants to prevent loss of the DNA pellets. DNA pellets were washed in 100% ethanol, air-dried and dissolved in 20 µl tris-EDTA buffer, pH 8.0, overnight (16 h). Reclaimed plasmid was stored at -20°C.

2.2.10 Production of electrocompetent E.coli MBM7070

LB broth (2 x 12 ml) was inoculated with a 1 ml aliquot of MBM7070 E.coli (15% glycerol in LB). The cultures were incubated for 16 h (overnight) in an orbital shaking incubator at 37°C and 250 rpm. The next morning the starter cultures were used to inoculate two 2 l flasks each containing 500 ml LB broth. These were incubated in an orbital shaking incubator (Gallenkamp, Germany) at 37°C and 250 rpm until an optical density of 0.5-0.7 at 600 nm was reached. The cells were chilled

on ice for 15 minutes then transferred to four 250 ml polypropylene copolymer centrifuge bottles. The tubes were centrifuged at 4200 rpm and 4°C in a Beckman J2-21m/E centrifuge using a JA-14 rotor (Beckman, Palo Alto, Ca., USA). The supernatant was removed and the pellets resuspended in 250 ml ice-cold sterile water. The cells were then centrifuged as before followed by another wash cycle (250 ml water). The pellets were then resuspended in 50 ml ice-cold 10% (v/v) glycerol solution. The cells were centrifuged at 4200 rpm and 4°C for 10 minutes. The supernatant was carefully removed and the pellets re-suspended in a pellets volume of 10% (v/v) glycerol. The cells were stored at -80°C in 100 µl aliquots ready for use.

2.2.12 High-efficiency transformation of E.coli *MBM7070 by electroporation*

pSP189 plasmid DNA (3 µl, recovered from human cells) was transferred to an aliquot of electrocompetent cells (100 µl, defrosted on ice) and mixed gently before being incubated on ice for 1 minute. The DNA-cell mixture was then transferred to a Biorad 0.2 cm gap electroporation cuvette, pre-cooled on ice. The DNA-cell mixture was electroporated at 2.5 kV, 25 µF and 200 Ω using Biorad Gene Pulser apparatus (time constant 4.2 ms) (Biorad, Hercules, Ca., USA). Immediately after electroporation 1 ml SOC medium was added. The mixture was transferred to a 13 ml sterile polypropylene tube and incubated for 30-45 minutes at 37°C and 250 rpm in an orbital shaker to allow expression of the ampicillin resistance phenotype.

2.2.13 Screening for mutant colonies

To calculate the number of viable plasmid containing cells per µl culture, small aliquots of transformed MBM7070 cells (10 µl, 25 µl, 50 µl, 75 µl) were plated out on 9 cm plates and grown overnight in darkness at 37°C (Figure 2.3.). Colonies were counted and numbers used to calculate how much to spread per 525 cm^2 bioassay dish (usually 300-500 µl). Any remaining transformed cells were stored at 4°C to be plated out at a later time. A counting marker pen was used to count colonies. Any white colonies (mutants) were noted and a mutation frequency was calculated.

Mutation Frequency = number of white colonies (mutant) / number of blue colonies (WT) + number of white colonies (mutant)

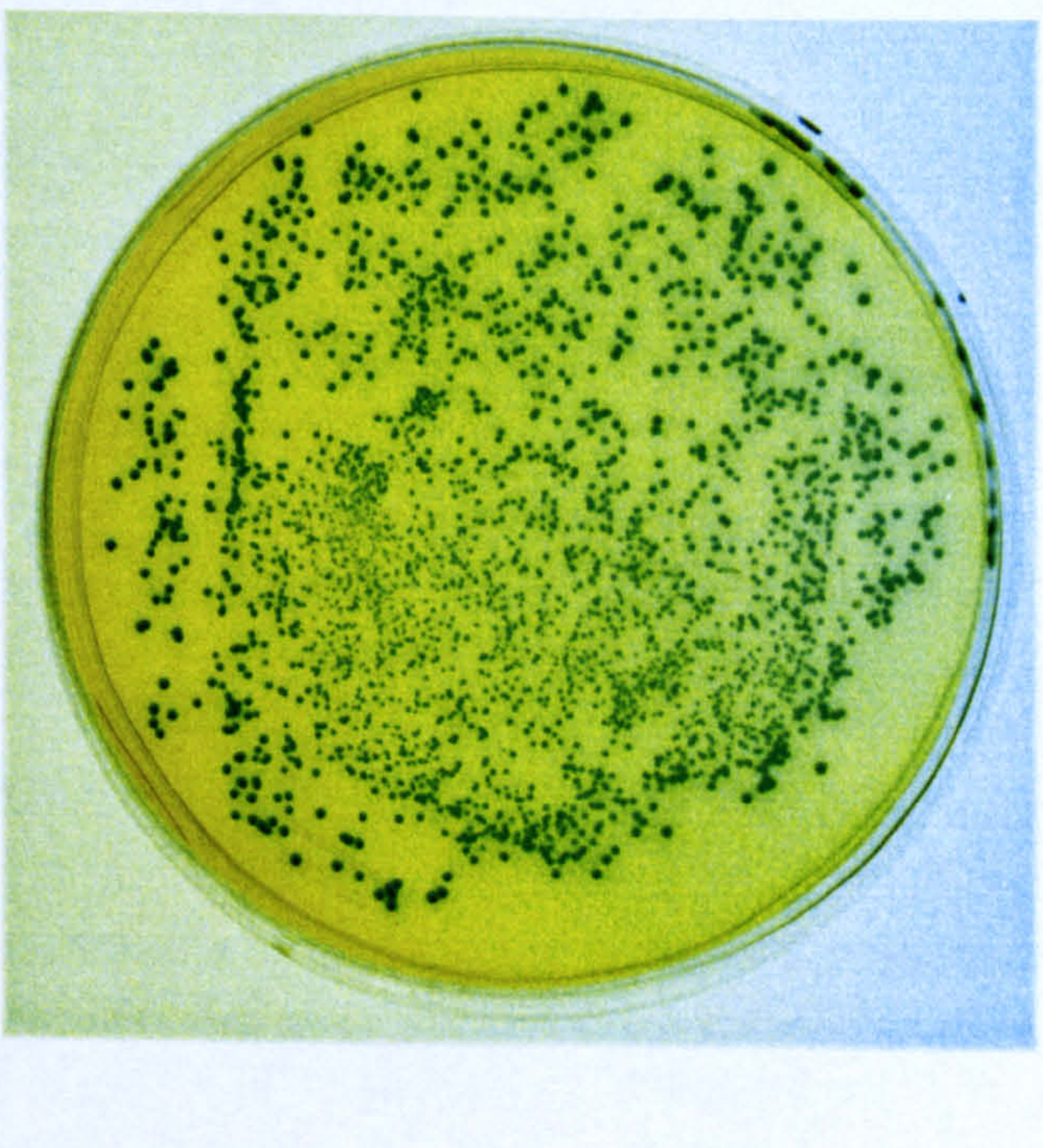


Figure 2.3. Illustrates a plate of blue MBM7070 *E.coli* colonies containing wild type non-mutant pSP189 plasmid.

Any white looking colonies were picked using a flamed wire loop and streaked out to single colonies on fresh 9 cm plates (25 μg/ml IPTG, 75 μg/ml X-gal, and 100 μg/ml ampicillin). Plates were grown in darkness for 16 h at 37°C. Single white colonies were re-streaked until it was possible to isolate single mutants (Figure 2.4.).

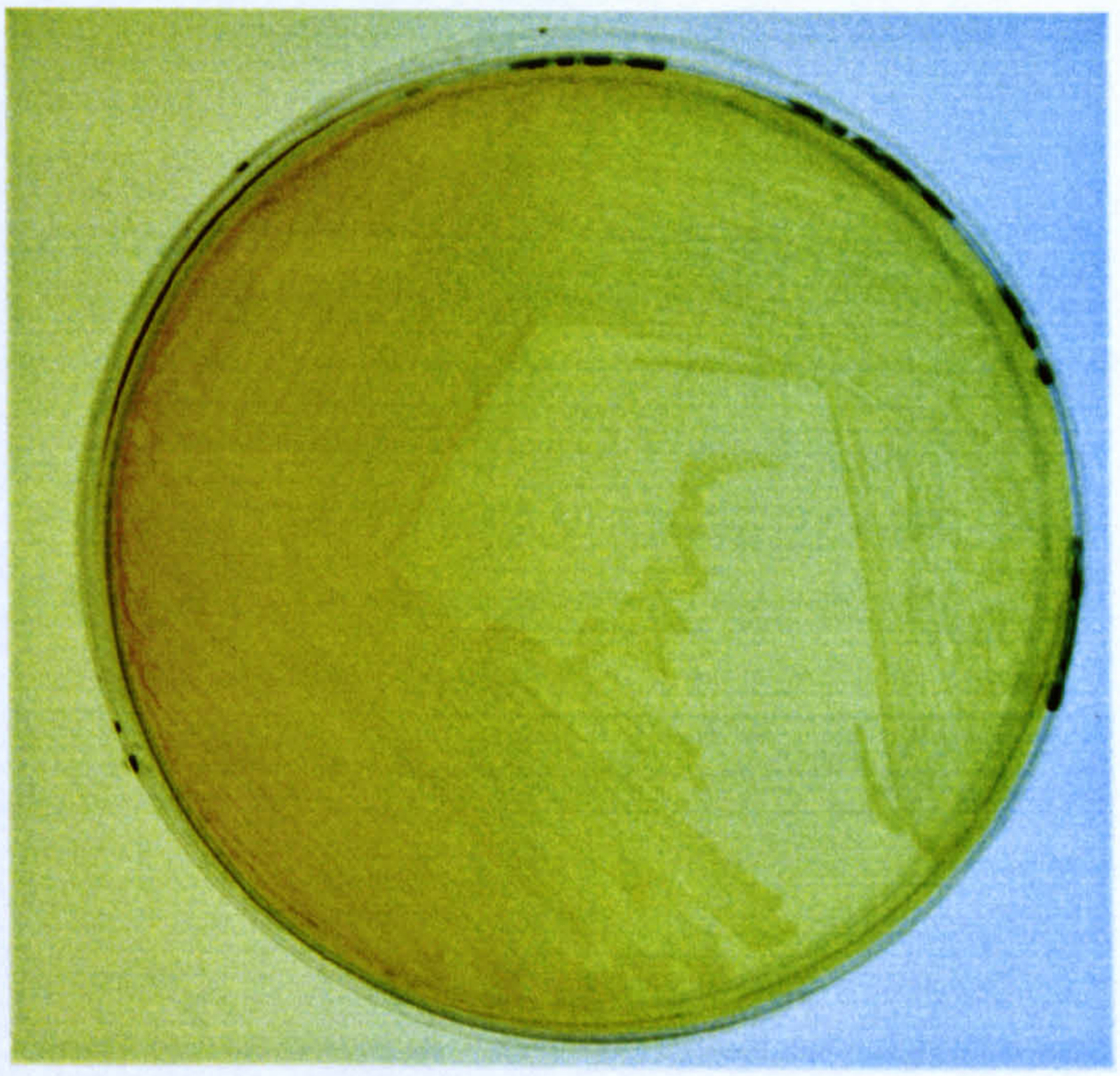


Figure 2.4. Illustrates a streak plate of white MBM7070 *E.coli* colonies containing mutant pSP189 plasmid.

2.2.14 Isolation of mutant pSP189 plasmid from agar plates

Single mutant colonies were picked, using sterile glass pasteur pipettes, and blown into 10 ml LB broth (100 μg/ml ampicillin), using sterile PVC teats. The colonies were grown for 16 h at 37°C and 250 rpm on a rotary shaking incubator (Gallenkamp, Germany). The cultures were then transferred to 100 mm ×16 mm round bottom tubes and pelletted, 3200 rpm, ≈2000 g, in a Beckman GPR centrifuge (Beckman,

Palo Alto, Ca., USA). Supernatants were removed and pellets sent to the Protein and Nucleic Acid Chemistry Laboratory (PNACL, University of Leicester, Leicester) for sequencing.

2.2.15 Plasmid sequencing

The optical density of the GGC GAC ACG GAA ATG TTG AA sequencing primer was measured at 260 nm. The DNA was precipitated by the addition of 5µl 3M sodium acetate and 100 µl ethanol per optical density unit (ODU). The oligonucleotide was briefly vortexed and incubated at 4°C overnight. The oligonucleotide was centrifuged for 5 minutes at 14000 rpm and 4°C and the supernatant removed. The pellet was washed with ethanol (100 µl per ODU) and respun, as before. The final supernatant was removed and the oligonucleotide dried by vacuum centrifugation (Savant Industries INC., Farmingdale, NY., USA). The oligonucleotide was dissolved in 100 µl sterile water and concentration calculated from its optical density at 260 nm. The sequencing primer was then diluted to 0.8 pmol/ µl and submitted at 10 µl per plasmid sample to be sequenced. Sequencing was carried out on an Applied Biosystems Model 377 DNA sequencer using BigDye version 1.0 sequencing chemistries. Oligonucleotide synthesis and DNA sequencing was carried out by the Protein and Nucleic Acid Laboratory (PNACL), University of Leicester)

2.3 Results

In order to assess the mutagenic effects of short wavelength UV radiation (UVC, 254 nm) the pSP189 shuttle vector plasmid was modified by *in vitro* treatment with three doses of UVC radiation (100, 300 and 600 J/m^2). Following replication in human adenovirus transformed kidney (Ad293) cells, recovered plasmid was used to transform MBM7070 indicator *E. coli*. Any plasmids containing a mutation in the *supF* gene grew as white colonies whilst non-mutant wild type plasmids grew as blue colonies.

2.3.1 Mutation frequency in supF *gene*

The spontaneous mutation frequency of plasmid treated with water (0.2×10^{-4}) was of a comparable value to those previously reported in Ad293 cells. Bigger, *et al.* (1990 and 1992) reported spontaneous mutation frequencies of 0.5 and 0.2×10^{-4}, whilst Boldt, *et al.* (1991) reported 0.8×10^{-4}. Mutation frequency increased with increasing dose of UVC radiation. Treatment of plasmid with UVC radiation resulted in a 5-fold increase in mutation frequency for the dose of 100 J/m^2 along with 7.5- and 26-fold with doses of 300 J/m^2 and 600 J/m^2, respectively (Table 2.1). Mutation frequency increases with dose with an R^2 value of 0.93, y=0.0081x - 0.0429 (graph not shown).

Table 2.1. Mutation frequency induced by ultraviolet radiation (254 nm)

Treatment	Colonies Screened	Number of Mutants	Mutation Frequency ($\times 10^{-4}$)
Control	322000	7	0.2
100 J/m^2	262000	27	1
300 J/m^2	200000	30	1.5
600 J/m^2	147000	77	5.2

2.3.2 Mutation types found in supF gene

Due to the low spontaneous mutation frequency induced by non-treated plasmid (only 7 mutants were found after sequencing), a mutation spectrum has been compiled from previously published *supF* studies using Ad293 cells (Bigger, *et al.*, 1990 and 1992; Boldt, *et al.*, 1991; and Juedes and Wogan, 1996). It was found that the majority of spontaneous mutations are in the form of single base substitutions (64% of all mutations, Table 2.2) followed by multiple mutations (21%) along with a small amount of frameshift mutations in the form of deletions. The spontaneous mutations seen in this study were three single base substitutions (two GC→TA transversions and one GC→AT transition), two multiple base deletions, a double base insertion and a nine base insertion.

The majority of mutations induced by UVC radiation were in the form of base substitutions (between 74 and 90% of all mutations) as shown in Table 2.2. At the lower dose (100 J/m^2) most of these were in the form of single base substitutions (67%) with a few multiple substitutions (7.4%, 2 or more substitutions at non-adjacent sites along the *supF* gene) and no tandem mutations (two adjacent substitutions). At 300 J/m^2 77% of all mutations were in the form of single base substitutions, with tandem mutations accounting for 13% of all mutations and no multiple substitutions.

The higher dose (600 J/m^2) induced 60% of all mutations in the form of single base substitutions, 10% in the form of tandem mutations and 7.8% in the form of multiple mutations.

Table 2.2. Types of sequence alterations in *supF* gene of pSP189 plasmids treated with ultraviolet radiation

Mutations	Number of plasmids with mutations (%)			
	Control[a]	100 J/m^2	300 J/m^2	600 J/m^2
Base substitutions	**63** (86)	**20** (74)	**27** (90)	**60** (78)
Single	47 (64)	18 (67)	23 (77)	46 (60)
Tandem	1 (1.4)	0 (0)	4 (13)	8 (10)
Multiple	15 (21)	2 (7.4)	0 (0)	6 (7.8)
Frameshifts	**10** (14)	**7** (26)	**3** (10)	**17** (22)
Single base deletion	4 (5.5)	1 (3.7)	0 (0)	2 (2.6)
>2 bases deletion	6 (8.2)	4 (15)	3 (10)	14 (18)
Single base insertion	0 (0)	1 (3.7)	0 (0)	0 (0)
>2 bases insertion	0 (0)	1 (3.7)	0 (0)	1 (1.3)
Total plasmids sequenced	**73** (100)	**27** (100)	**30** (100)	**77** (100)

[a] adapted from Bigger, *et al.*, 1990 and 1992; and Juedes and Wogan, 1991.

As can be seen in Table 2.3 and Figure 2.5, there is a distinct difference in the pattern of base substitution types observed after treatment with UVC radiation compared to the control mutations. In the spontaneous control spectrum the major substitution is the GC→TA transversion (51% of all single base substitutions), occuring over 2-fold more frequently than GC→CG transversions (20%) and GC→AT transitions (18%). AT→TA transversions (6%) and AT→CG transversions (4%) were seen but no AT→GC transitions. In the mutation spectra induced by treatment with UVC radiation the major substitution is the GC→AT transition (61%, 87% and 76% of all single base substitutions for 100 J/m^2, 300 J/m^2 and 600 J/m^2, respectively). This mutation is over 5-fold more prevalent than any other substitution mutation. A very low level of transversions (between 0% and 14%) and AT→GC transitions (between 3 and 11%) are also seen after UVC treatment.

Table 2.3. Types of single base substitution mutations in *supF* gene of pSP189 plasmids treated with ultraviolet radiation

Mutations	Number of plasmids with mutations (%)			
	Control	100 J/m^2	300 J/m^2	600 J/m^2
Transversions	**40** (82)	**5** (28)	**3** (10)	**12** (19)
GC→TA	25 (51)	1 (6)	2 (6)	9 (14)
GC→CG	10 (20)	2 (11)	1 (3)	1 (1.6)
AT→TA	3 (6)	2 (11)	0 (0)	1 (1.6)
AT→CG	2 (4)	0 (0)	0 (0)	1 (1.6)
Transitions	**9** (18)	**13** (72)	**28** (90)	**51** (81)
GC→AT	9 (18)	11 (61)	27 (87)	48 (76)
AT→GC	0 (0)	2 (11)	1 (3.2)	3 (5)
Total single base substitutions	**49** (100)	**18** (100)	**31** (100)	**63** (100)

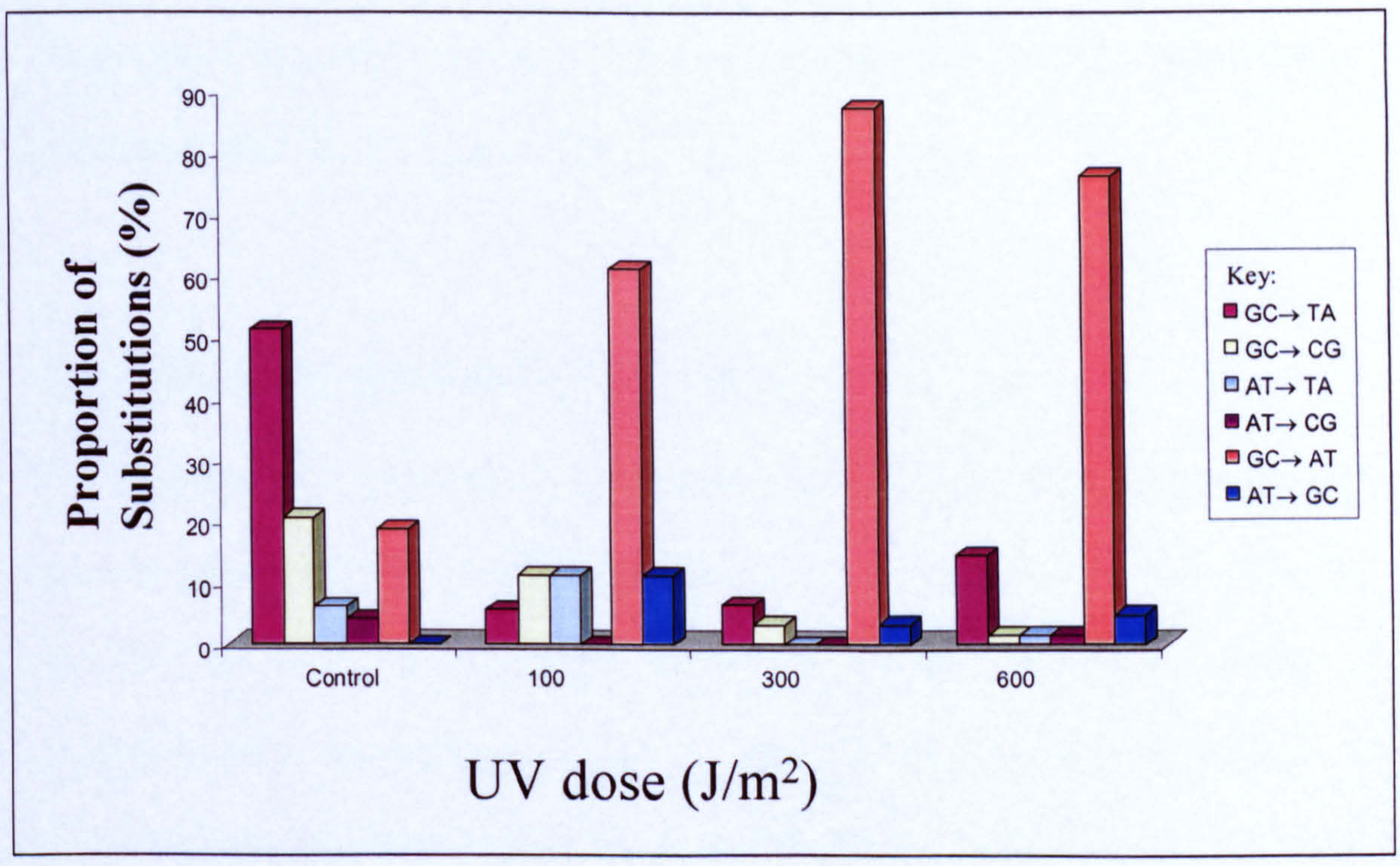


Figure 2.5 Bar chart illustrating the proportion of the different single base substitution mutations seen in control plasmids and in those induced by treatment with UVC (254 nm) radiation at doses of 100 J/m^2, 300 J/m^2 and 600 J/m^2.

All deletions, apart from three, began at GC base pairs (21 out of 24, 88%, Figure 2.6). Of the three insertions two were at GC bases. The size of deletion varied from

one base to greater than 71 bases. In no case was the whole *supF* gene deleted but larger deletions were seen to start or end before or after the *supF* gene coding region.

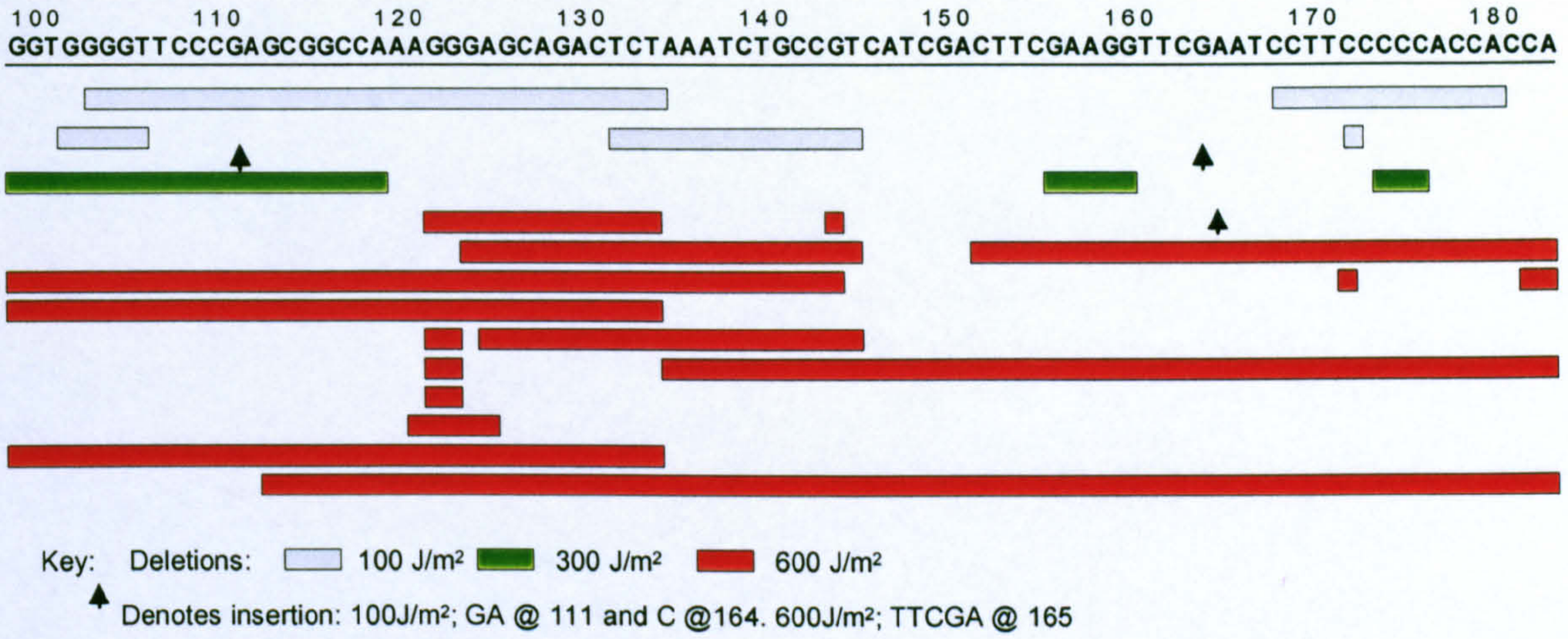
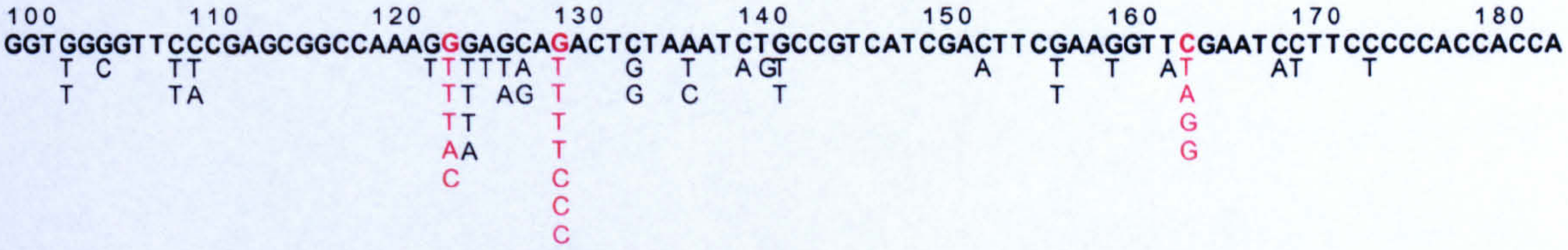


Figure 2.6. Frame shift mutations induced by treatment with UVC (254 nm) radiation at doses of 100 J/m^2, 300 J/m^2 and 600 J/m^2.

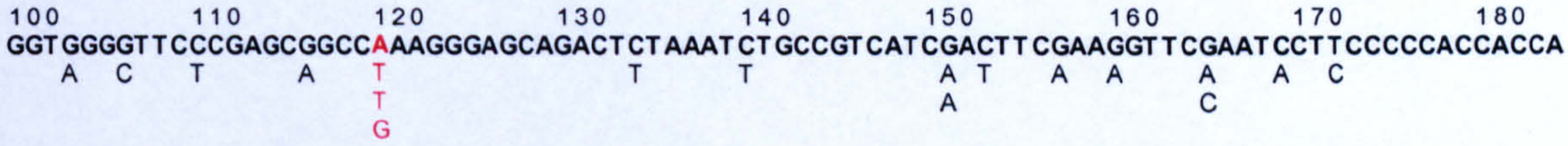
2.3.3 Mutation spectra in supF gene

The distribution of single base and tandem substitution mutations within the *supF* gene for plasmids dosed with UVC (254 nm) radiation at doses of 100 J/m^2, 300 J/m^2 and 600 J/m^2 or control treatments is shown in Figure 2.7. When these spectra are compared using the hyperg program (a computer program for the analysis of mutational spectra; Cariello, *et al.,* 1994), treated spectra are found to be significantly different from control (p (same) $\leq$ 0.05). Mutations are not distributed randomly, but concentrated at one or more sites, known as hotspots. A hotspot was defined as a site where the number of mutations observed was 4-fold or more, greater than the number expected for a random (Poisson) distribution.

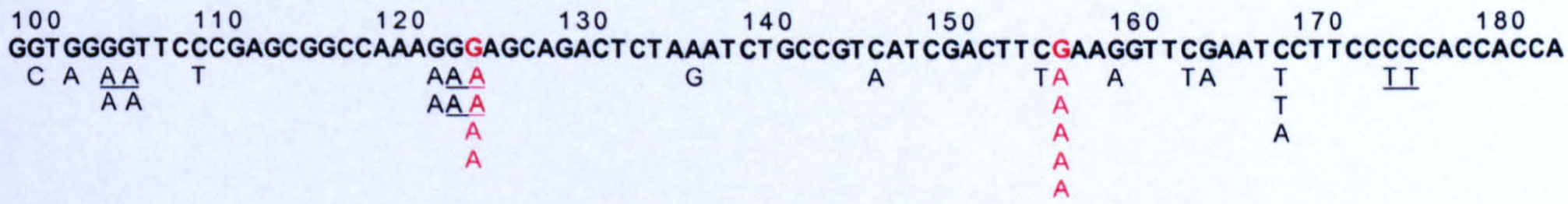
Spontaneous Mutations



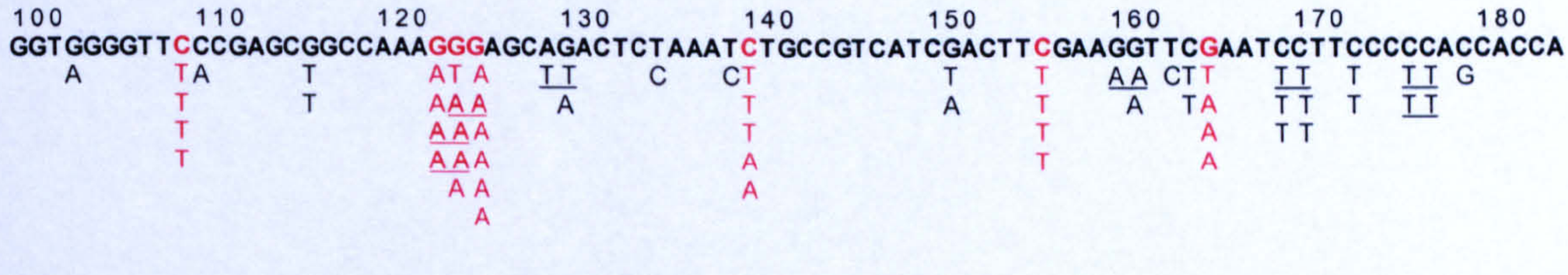
UVC 100J/m²



UVC 300J/m²



UVC 600J/m²



UVC all doses combined

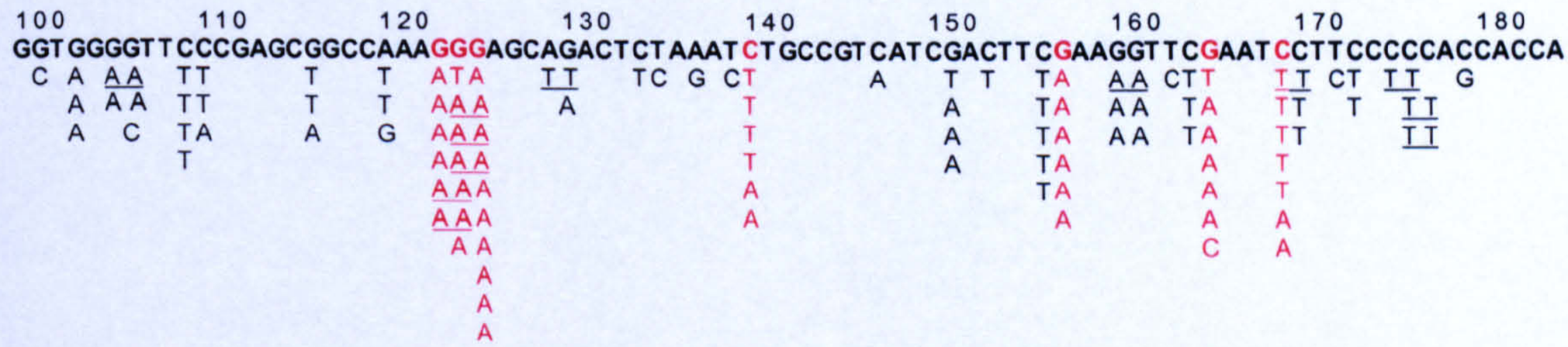


Figure 2.7. Mutation spectra depicting base substitutions induced spontaneously and after treatment with 100 J/m², 300 J/m² and 600 J/m² UVC radiation. Spontaneous control spectrum taken from Bigger, *et al.,* (1990 and 1992), Boldt, *et al.,* (1991) and Jeudes and Wogan (1996). Hotspots are shown in red.

The distribution of spontaneous mutations has three hotspots in its mutation spectrum at positions 123, 129 and 163 all of which are at GC basepairs (Bigger, *et al.*, 1990 and 1992; Boldt, *et al.*, 1991; and Juedes and Wogan, 1996)(Figure 2.7). UVC

irradiation of plasmid at 100 J/m^2 induces hotspots at a single AT basepair of position 119. Increasing dose of UVC irradiation increases the number of hotspots seen in the *supF* region of the plasmid. At 300 J/m^2 two hotspots are seen (positions 124, and 156), whilst at 600 J/m^2 seven hotpots are induced at positions 108, 122, 123, 124, 139, 155 and 164. If all treatments are combined seven hotspots are seen at positions 122, 123, 124, 139, 156, 164, and 168. All, bar one, hotspots induced by UVC irradiation of the pSP189 plasmid are at positions of GC basepairs.

2.4 Discussion

This chapter presents a comparison of the patterns of mutagenesis induced in the *supF* gene by different doses of UVC radiation. Since UV mutagenesis has been widely studied in the context of the *supF* assay, both in human and monkey cells (Bredberg, *et al.,* 1986, Madzak, *et al.,* 1992), the primary aim of this study was to evaluate and validate the *supF* assay as a tool for future mutagenesis investigations in our laboratory. pSP189 plasmid DNA was treated with UVC light (254 nm wavelength) to induce DNA damage. The doses used were 100 J/m^2, 300 J/m^2 and 600 J/m^2 along with a control treatment without UV irradiation. This study investigates the mutation spectra induced when treated plasmid was replicated in human adenovirus transformed kidney (Ad293) cells followed by screening in MBM7070 *E.coli.* These possess an amber mutation in the *lacZ* (β-Galactosidase) gene allowing the *supF* containing plasmid to produce blue wild type colonies when grown on 5-bromo-4-chloro-3-indolyl-β-D-galactose (X-gal) containing plates. Any mutations in the *supF* gene will result in white colonies. Mutant colonies were grown and sequenced to yield a distribution of mutations induced by the three doses of UVC radiation

Treatment of pSP189 plasmid with UVC (254 nm) radiation resulted in an increase in mutation frequency in the *supF* gene by up to 26-fold over control. All three UVC treatments increased mutation frequency over control, and when plotted on a graph the mutation frequency was found to increase almost linearly with dose ($R^2 = 0.93$). Mutation frequencies for the treated plasmids were of comparable magnitude (up to 5.2×10^{-4} for 600 J/m^2 dose) to those seen before in this cell line by various chemical mutagens and carcinogens. Treatment of the pSP189 plasmid with 0.1 M sodium

nitrite pH 5.4 or 1 M sodium nitrite pH 6.4 induced mutation frequencies of 5.5×10^{-4} and 4.3×10^{-4} in Ad293 cells, respectively (Routledge *et al.*, 1994). A dose of 60 ng of the *anti* 1,2-dihydrodiol 3,4-epoxide of 5-methylchrysene, the environmental carcinogen, gave a mutation frequency of 5.07×10^{-4} in Ad293 cells (Bigger, *et al.*, 1990). When stereoisomers of benzo[*c*]phenanthrene dihydrodiol epoxide (B*c*PhDE) were investigated in the same system doses of up to 160 ng/ml induced mutation frequencies of a comparable magnitude (in the range, $1.6\text{-}7.5\times10^{-4}$) (Bigger, *et al.*, 1992). Previous investigations using UV radiation to induce mutations in the *supF* gene gave mutation frequencies which were one order of magnitude higher than those reported here, however these were replicated in different cell lines and used different plasmid derivatives. Bredberg, *et al* (1986) dosed pZ189 plasmid with 254 nm radiation in both *Xeroderma pigmentosum* derived XP12BE(SV40) cells and their normal GM0637(SV40) counterparts. Mutation frequencies of up to 7×10^{-3} at a dose of 300 J/m^2 were seen in XP cells and up to 6.4×10^{-3} at a dose of 500 J/m^2 in normal cells. UVC (254 nm) dosed plasmid replicated in CV-1 African green monkey cells induced a mutation frequency of 7.2×10^{-3} at a dose of 500 J/m^2, although the spontaneous mutation frequency was also an order of magnitude higher (5×10^{-4}) (Keyse, *et al.*, 1988).

All possible mutation types were seen in the treated plasmids. These were either, the four possible transversions (GC→TA, GC→CG, AT→TA or AT→CG), two possible transitions (GC→AT or AT→GC) or any frameshift deletions or insertions. In fact a full range of deletions was seen; varying from single base pairs being removed to long stretches of up to 71 basepairs. The distribution of mutations was similar for all three doses of UVC radiation given. There was a preference for base substitution mutations

which was also seen previously (Bredberg *et al.*, 1986) after UVC induced mutagenesis in the *supF* gene. These substitution mutations were preferentially seen at GC base pairs generally as GC→AT transitions which has also been noted before (Bredberg, *et al.*, 1986, Keyse, *et al.*, 1988, Parris and Seidman, 1992; Madzak, *et al.*, 1992). This is a contrast from the mutations seen in the combined spontaneous spectrum, where the preference is for GC→TA transversions. In a previous study looking at the respective roles of the different UV photoproducts it was suggested that cytosine containing cyclobutane pyrimidine dimers or (6-4') photoproducts were the premutagenic lesions which induced GC→AT transitions, and (6-4') photoproducts but not thymidine containing cyclobutane dimers were the premutagenic lesions responsible for AT→GC transitions (Otoshi, *et al.*, 2000). In this study it was then deduced that the major lesion induced by UVC irradiation of the *supF* gene was in the form of a pyrimidine dimer containing at least one cytosine.

Looking at the frameshift mutations induced by UV irradiation, it can be noted that these begin at sites where cyclobutane pyrimidine dimers or (6-4') photoproducts could be produced (i.e. at sites of two or more adjacent pyrimidines). The proportion of frameshifts (up to 26%) is higher than was seen in XP and normal cells (Bredberg, *et al.*, 1986) and in COS-7 cells (Madzak, *et al.*, 1992) but lower than those seen in CV-1 cells (Keyse, *et al.*, 1988)

Looking at the sites of preferential mutagenesis through the *supF* gene it can be seen that UVC radiation only induces hotspots at sites where cyclobutane pyrimidine dimers or (6-4') photoproducts could be produced. The hotspots appear at cytosine rather than thymidine in nine cases out of ten, in keeping with the principle of

cytosine containing dimers being more mutagenic (Otoshi, *et al.*, 2000). The mutations seen at hotspot sites were generally transitions.

2.4.1 Summary

The aim of this study was to successfully validate the *supF* forward mutation assay in our laboratory. This has been shown using UV radiation. Results presented here do not totally match those seen before, although this may be due to the fact that different cell lines and plasmids have been used. These results do show a relationship between UV dose and mutation frequency and also show that the most prevalent mutation, the GC→TA transition, was that already proved to be important in UV induced mutagenesis.

Chapter 3

Mutations induced in the pSP189 *supF* gene by benzo[*a*]pyrene diol epoxide (BPDE) and ultraviolet radiation

3.1 Introduction

3.1.1 Benzo[a]pyrene

The English surgeon Percival Pott is regarded by many as the first to investigate chemical carcinogenesis. He observed that an environmental agent was responsible for tumour induction in chimney sweeps in London (Pott, 1775). Chimney sweeps in those times were exposed to very high levels of polycyclic aromatic hydrocarbons derived from the combustion of coal in the presence of air. In a series of papers, he characterised a high incidence of scrotal cancer associated with this occupation and suggested that deposits of soot in the scrotal area were causative. As a result of this, the Danish chimney sweepers' guild urged its members to bathe daily, and by 1892 a lower incidence of scrotal cancer was noted in Northern Europe in comparison to England (Butlin, 1892). This had almost eliminated scrotal cancer as an occupational hazard. This case clearly demonstrated not only that an environmental substance could induce tumour formation but also that by identifying and eliminating the aetiologic agent from the immediate environment, tumour incidence could be significantly reduced.

It was later established that there was an association between exposures to soot and cancer incidence for a number of other professions, such as gardening (Earle, 1808) and tar manufacturing (Bell, 1794). More recently the crude oils used in cotton spinning (Heller, 1930) and tool setting (Waldron, *et al.*, 1984) were reported to cause scrotal cancer in workers, as their trousers tended to become saturated in oils. A century passed between these first observations and the use of animal models to test the idea that chemicals can cause tumours and to permit the purification of the

carcinogens responsible. In 1918 Yamagawa and Ichikawa demonstrated that the chronic application of coal tar to the ears of rabbits could induce skin carcinomas. Chemists could now examine the carcinogenicity of purified fractions from tar products. Their studies established a clear multistage process consisting of early irritation and proliferation of cells, papilloma formation at the site of application, neoplasia, and even metastasis in several animals. The successful extraction of a crystalline substance responsible for the carcinogenicity of coal tar on rabbit skin was described by Cook, *et al.* (1933). The compound, benzo[*a*]pyrene (B[*a*]P) was classified as a member of a family of carcinogens known as polycyclic aromatic hydrocarbons (PAH). It has since been studied extensively as a metabolism dependent chemical carcinogen and has proven invaluable in elucidating the mechanisms for carcinogen activation.

Since the discovery of B[*a*]P, hundreds of other occupational and environmental chemical carcinogens have been identified. These include a variety of organic substances (e.g. PAH and aromatic amines), as well as a number of environmentally important metals and minerals, such as nickel and asbestos. More than 95% of these organic chemicals fall into one of three categories: alkylating agents, aralkylating agents, or arylamines. These agents differ in the type of group to be transferred to form a DNA adduct, but they share certain characteristics. They are either intrinsically reactive with DNA, or can be metabolized to form DNA active intermediates. These, so called, electrophiles bond with the electron-sharing atoms of the DNA nucleotides, such as ring and exocyclic nitrogen or exocyclic oxygen atoms, to form stable altered nucleotides, or adducts (Hemminki, 1994). Despite the identification of large number of other carcinogens from industrial or environmental

origins, B[*a*]P remains to this day one of the most highly carcinogenic compounds known. Exposure to coal tar is not the public health hazard it was at the time of the industrial revolution, nevertheless exposure to B[*a*]P from sources such as cigarette smoke and automobile exhausts is still highly prevalent in the environment (Albert and Burns, 1977). The development of ^{32}P- postlabelling procedures (Gupta, *et al.*, 1982; Gupta, 1985) has allowed for the detection of B[*a*]P (and other PAH) adducts in biological samples. Another method, ^{14}C-postlabelling coupled with accelerator mass spectrometry (AMS) (Goldman, *et al.*, 2000) has been developed by investigating B[*a*]P modified DNA *in vitro*. Such methods have become useful tools in the investigation of the genotoxicity of environmental and industrial carcinogens and their relative cancer risk to humans.

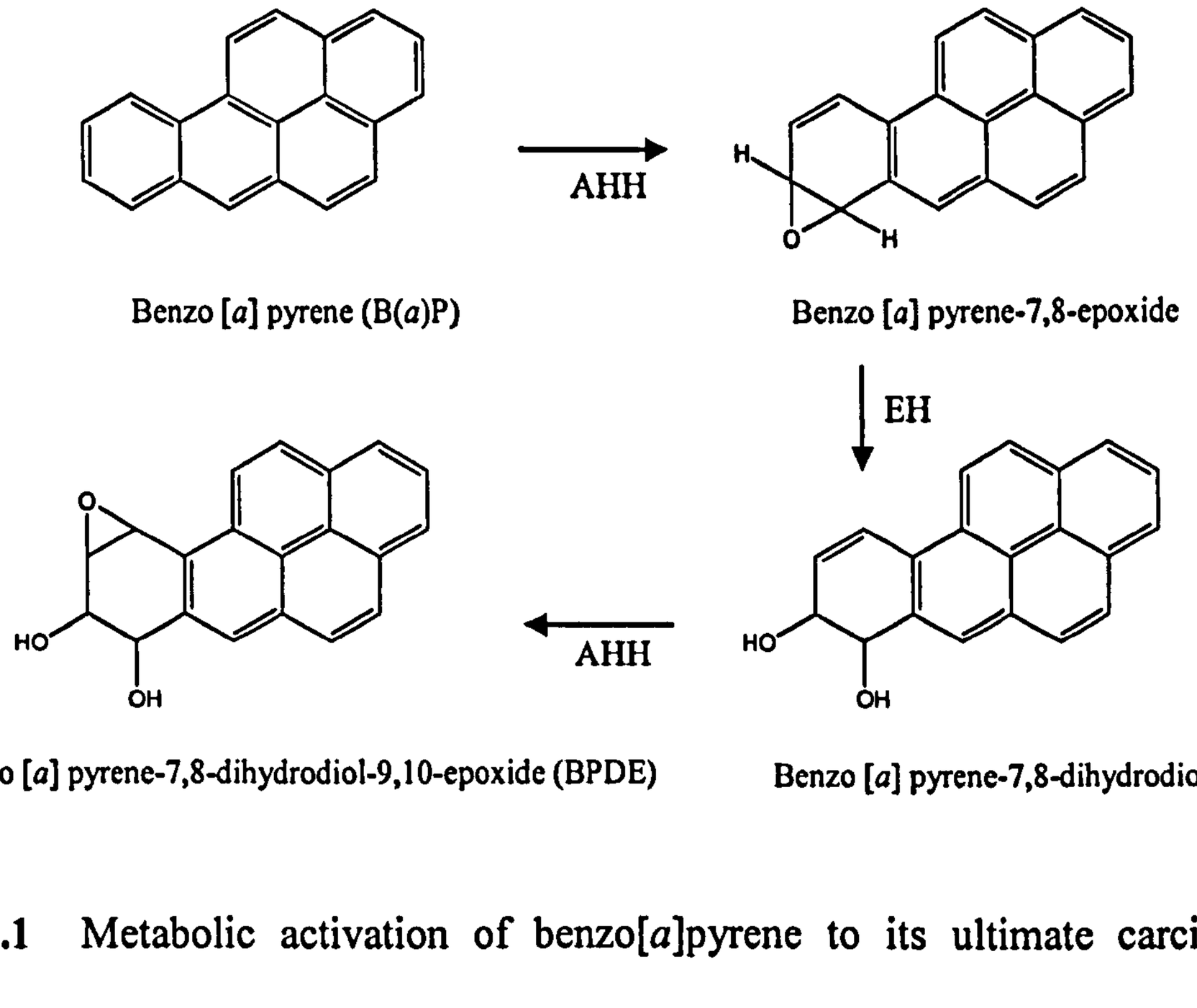


Figure 3.1 Metabolic activation of benzo[*a*]pyrene to its ultimate carcinogen, benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide. Activation is mediated by the P-450 mixed function oxidase system, specifically arylhydrocarbon hydroxylase (AHH) and epoxide hydratase (EP).

Unmodified B[*a*]P is an unreactive nonpolar compound with a planar configuration (Figure 3.1). This configuration allows for its intercalation between the hydrogen bonded base pairs in duplex DNA, and it was this factor which was initially thought to be the likely mode of DNA damage by this compound. It has since been established that B[*a*]P and other PAHs are metabolised by components of the P-450 mixed function oxidase system known as arylhydrocarbon hydroxylases (AHH) (Hall and Grover, 1990; Levin, *et al.*, 1977; Phillips, 1990; Selkirk, *et al.*, 1982). Metabolism of B[*a*]P to phenolic compounds (1, 3, 6, or 9-OH's), or with the addition of dihydrodiol groups (4,5-diol or 9,10 diol), allows for the excretion of the compound, along with their corresponding ester conjugates. However, some of the products of B[*a*]P metabolism are electrophilic epoxides, and it is well established that the ultimate carcinogenic form of this compound is the *anti* diol epoxide called BPDE (Hall and Grover, 1990; Selkirk, *et al.*, 1982)(Figure 3.1). This is produced after two rounds of oxidation with arylhydrocarbon hydroxylases, with an intermediate step where the 7,8-epoxide is further oxidized by the enzyme epoxide hydratase (EP) (Figure 3.1). The intermediates are sufficiently stable to allow their passage to the cell nucleus but also reactive enough to form adducts with DNA (Jerina, *et al.*, 1991).

Following the noncovalent interaction of intercalation with DNA (Geacintov, 1986), the C-10 position of the benzo(*a*)pyrene *anti* diol-epoxide (BPDE) binds predominantly at the exocyclic 2-amino position of guanine (Cosman, *et al.*, 1992; Gräslund and Jernström, 1989; Selkirk, *et al.*, 1982; Weinstein, *et al.*, 1976) (Figure 3.2). This adduct in a CGC sequence context has been purified and nuclear magnetic resonance spectroscopy has shown that it causes only minimal perturbation of the B-DNA helix in duplex DNA, with the B[*a*]P rings being positioned in the minor groove

(Cosman, *et al.*, 1992). BPDE reacts non randomly with DNA as it has a preference to react with guanines (Boles and Hogan, 1986; Thrall, *et al.*, 1992) especially if the guanine is flanked by another guanine on the same strand (Osborne, 1990).

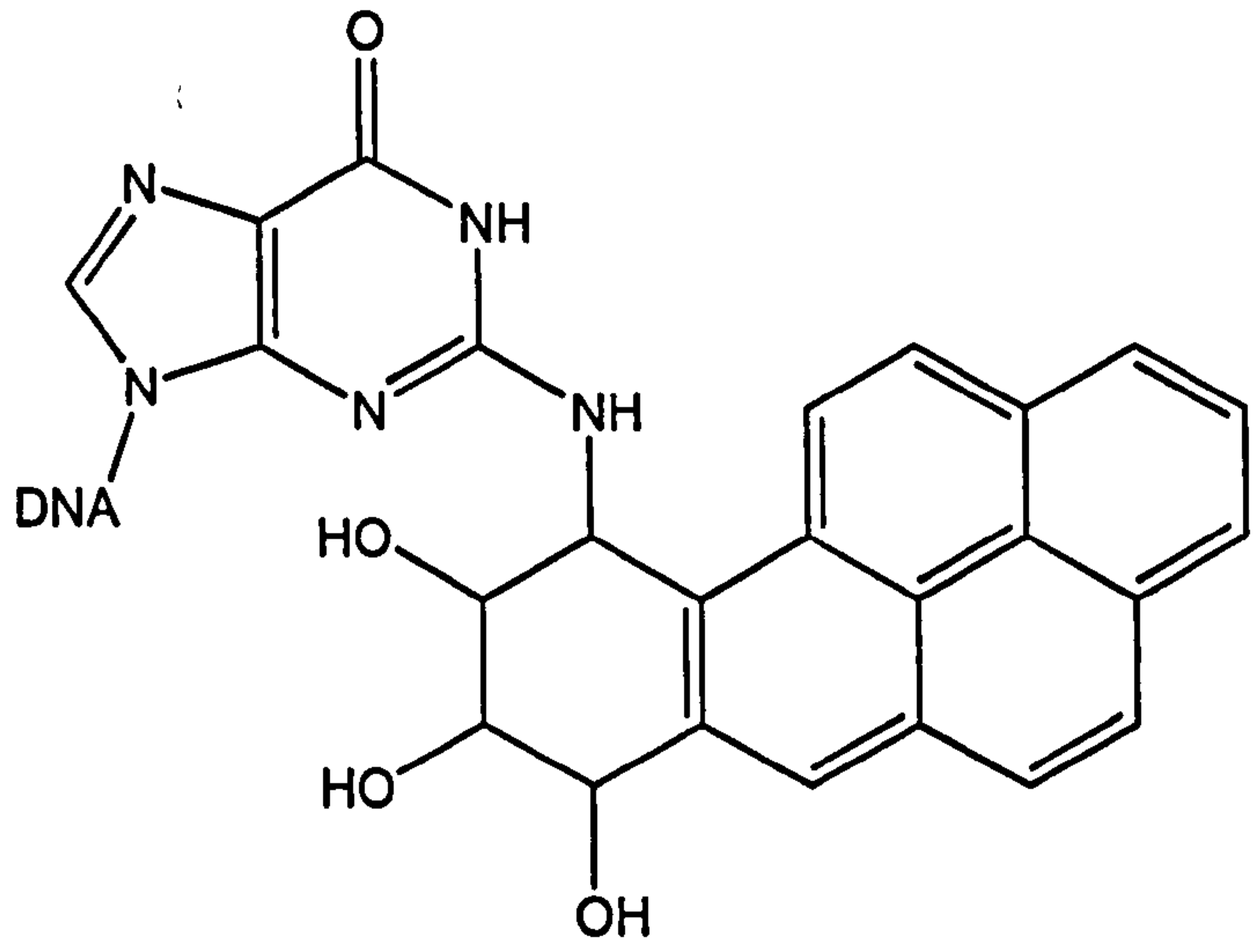


Figure 3.2 The major benzo[*a*]pyrene DNA adduct formed by the reaction of benzo[*a*]pyrene diol epoxide with the exocyclic amino group of deoxyguanosine.

Many studies have been performed looking at the mutagenesis induced by the reaction of BPDE and DNA. In mouse cells, using a shuttle vector carrying the *supF* gene, it was found that the major mutation induced by BPDE was the GC→TA transversion (Courtemanche and Anderson, 1999). This was also concluded after BPDE treated plasmid was replicated in Ad293 cells (Yang, *et al.*, 1987). In a polymerase arrest study, looking at the distribution of BPDE DNA-lesions in pS189 plasmid containing the *supF* gene, it was found that BPDE preferentially bound to guanosine residues (69% of all stop sites), and adenosine (17%) with cytosine and thymidine accounting for 9% and 4%, respectively (Ross, *et al.*, 1994). Investigations looking at the site selectivity of the BPDE deoxyadenosine adduct in single stranded M13 vector found

substantial numbers of AT→TA transversions (Page, *et al.*, 1999). Mutagenic replication in human HeLa cell extracts, however, shows that the major mutation induced by the BPDE deoxyadenosine adduct is the AT→GC transition (Lavrukhin and Lloyd, 1998). BPDE also induces a dose dependent increase in mutation frequency of the hypoxanthine (guanine) phosphoribosyltransferase (*HPRT)* gene in *XPA* deficient mice (Bol, *et al.*, 1998) which suggests a role for the nucleotide excision repair mechanism in BPDE DNA adduct repair and mutagenesis. BPDE has also been shown to predominantly induce GC→TA transversions in the *HPRT* gene in diploid human fibroblasts (Chen, *et al.*, 1990). This investigation also alluded to a preference for repair of BPDE adducts on the transcribed DNA strand. Investigations have not been limited to mammalian and bacterial systems; B[*a*]P also preferentially induces transversions at GC basepairs in the *rpsL* gene of transgenic zebrafish embryos (Amanuma, *et al.*, 2001).

3.1.2 Ultraviolet radiation

A full introduction to ultraviolet (UV) radiation is given in Chapter 2. UVB induces the symptoms of sunburn and adaptive responses to exposure manifest themselves as hyperpigmentation and skin thickening. The shorter wavelength UVC radiation is mainly absorbed by the stratosphere, by reacting with ozone, or clothing and layers of dead skin, so gives little cause for concern over health issues. The biological role of UVA radiation is not fully understood. UVA exposure levels do not vary with time of day or season which may suggest a lack of association with skin cancer induction although there are indications that it can affect tissue injury in conjunction with UVB and certain photosensitizing chemicals (Urbach, 1993).

UV radiation has been implicated in the induction of non-cutaneous carcinomas, the most common and generally the most easily treated forms of cancer (Urbach, 1993; Marks, 1996), along with highly aggressive cutaneous melanomas (Lee and Strickland, 1980; Garland, *et al.*, 1990). UV radiation induces the formation of pyrimidine cyclobutane dimers (Beukers and Berends, 1960) and pyrimidine-pyrimidone (6-4) photoproducts (Lippke, *et al.*, 1981) both of which are formed between adjacent pyrimidine bases. The involvement of dimer formation in carcinogenesis is strongly supported by studies of the genetic defect *Xeroderma pigmentosum*, a complex of disorders characterised by deficient excision repair of UV-induced pyrimidine dimers and a high incidence of skin cancer (Kraemer, *et al.*, 1987, also see chapter 1). *In vitro* studies have shown that the UV action spectrum for transformation of hamster embryo cells (Doniger, *et al.*, 1981) or human embryonic fibroblasts (Sutherland, *et al.*, 1981), is consistent with that for UV induced dimer formation. The role of UV induced mutagenesis in the *supF* gene is discussed in Chapter 2.

3.1.3 Complex Mixtures of Carcinogens or Mutagens

In order to elucidate the mechanisms involved in mutagenic processes studies are usually performed using single agents. However, humans are naturally exposed to complex mixtures of carcinogens in the environment and combinations of mutagens, e.g., cigarette smoke or diesel exhaust emissions (Vainio, *et al.*, 1990). Induction of damage and mutations by an individual agent may be altered if the exposure occurs in combination with another agent or as a mixture of agents. For example, it has been demonstrated that some carcinogenic compounds that normally require metabolic activation to reactive species before binding to DNA occurs, can be photoactivated to

direct acting mutagens by UVA irradiation (McCoy, *et al.*, 1979; Israel-Kalinsky, *et al.*, 1982; Arimoto-Kobayashi, *et al.*, 1997). As well as interactions between agents prior to DNA binding, which may influence either the binding of genotoxic agents or the mutagenicity of the reactive species, the mutagenicity of the actual DNA adducts may be modified by other DNA reactive agents, or by the presence of other lesions on the DNA molecule.

Evidence that exposure to PAH and UV radiation, in the form of sunlight, may have a combined toxic effect *in vivo* comes from studies investigating the phototoxicity of PAH in aquatic organisms (Nikkila, *et al.*, 1999; Boese, *et al.*, 2000; Laycock, *et al.*, 2000). This combined effect may have implications in human carcinogenesis in situations where occupational exposure to PAH is combined with regular exposure to high levels of sunlight, such as for road maintenance workers (Hong and Lee, 1999) or when coal tar applications are used in combination with UV irradiation as a treatment for psoriasis (Pion, *et al.*, 1995).

3.1.4 Aim

This chapter describes an investigation into the effects of binary mixtures of carcinogens on the mutagenesis of the *supF* gene in the pSP189 plasmid shuttle vector. pSP189 plasmid was irradiated with UVB (302 nm) or UVC (254 nm) light before or after treatment with BPDE. Treated plasmid was transfected into human adenovirus transformed kidney (Ad293) cells and allowed to replicate. Plasmid was extracted after a suitable time and used to transform the MBM7070 screening bacteria. Any DNA damage induced by the complex treatments that has not been repaired, or has been mis-repaired, will result in a non-viable *supF* gene product, and

no suppression of the *lacZ* mutation. This will lead to the growth of white mutant colonies on plates containing the lactose analogue, 5-bromo-4-chloro-3-indolyl-β-D-galactose (X-gal).

3.2. Materials and Methods

Materials

All chemicals were from Sigma-Aldrich, Poole, Dorset, UK unless otherwise stated.

Methods

All methods specific to this chapter are described below. For any other procedures please refer to Chapter 2.

3.2.1 Treatment of pSP189 plasmid with BPDE followed by UVB or UVC irradiation

Plasmid DNA (100 μg in 50 μl water) was incubated in darkness with either acetone (10 μl) or BPDE dissolved in 10 μl acetone (~10 μM) for 15 min at 37°C. Unreacted BPDE was removed by extraction into ethyl acetate. DNA was precipitated by addition of 0.1 volumes 2.5 M sodium acetate, pH 5 and 2.5 volumes of ethanol, followed by storage at –20°C, overnight. Precipitated DNA was pelleted and washed twice with 70 % (v/v) ethanol. The pellet was dried and re-dissolved in 50 μl sterile, tissue culture grade water. Aliquots (10 μl) from control and treated plasmid were treated with UVB (302 nm) or UVC (254 nm) irradiation. For UVB irradiation, the plasmid samples were irradiated with an intensity of 0.2 mW/cm^2 for 20 min (giving a dose of 2.4 kJ/m^2), whilst for UVC irradiation, the plasmid samples were irradiated with an intensity of 0.7 mW/cm^2 for 1.5 min (giving a dose of 0.63 kJ/m^2). These doses were chosen as they induce similar levels of conversion of supercoiled to

relaxed plasmid in a plasmid single strand break assay (Routledge, *et al.*, 2001). After irradiation, plasmid samples were stored at 4°C until transfection into the Ad293 cells, which occurred within 24 hours of the irradiation.

3.2.2 Treatment of pSP189 plasmid with BPDE preceded by UVB or UVC irradiation

Aliquots of plasmid DNA (100 μg in 50 μl water) were irradiated with either UVB (302 nm) or UVC (254 nm), followed by treatment with BPDE as described before (Section 3.2.1). Briefly, plasmid DNA (100 μg in 50 μl water) was incubated, in darkness, with either acetone or BPDE (~10 μM). Unreacted BPDE was removed, the DNA precipitated and washed. The pellets were dried and re-dissolved in 100μl sterile tissue culture grade water. After treatment plasmid samples were stored at -20°C until transfection into the Ad293 cells.

3.2.3 ^{32}P-Postlabelling of BPDE treated pSP189 plasmid DNA

3.2.3.1 Digestion

The method is outlined in Chapter 1, Figure 1.8. BPDE, UVB and UVC treated pSP189 plasmid and control plasmid samples (1 μg of each) were dried by vacuum centrifugation in 1.5 ml ultra-centrifuge tubes. To each tube was added 10 μl digestion mixture. The digestion mixture contained 175 mU micrococcal nuclease, 3 mU calf spleen phosphodiesterase, 1 μl SSCC buffer (100 mM sodium succinate and 50 mM calcium chloride) and sterile water to make up to 10 μl. Samples were incubated for 3 h at 37°C.

3.2.3.2 Radiolabelling of adducted nucleotides

Aliquots (3×1 µl {0.1 µg DNA}) were removed from the digestion mixture and samples were transferred to a perspex box and labelled with 9 µl labelling mixture. The labelling mixture contained 20 µl labelling buffer (200 mM Tris HCl, 100 mM magnesium chloride, 100 mM DTT, 10 mM spermidine, filter sterilised), 6 µl T4 polynucleotide kinase (Roche Diagnostics Ltd., Lewes, East Sussex, UK), 20 µl ^{32}P-radiolabelled ATP{7.4 MBq [γ-^{32}P]ATP (Amersham Life Sciences Ltd, Little Chalfont, Bucks., UK)}, 20 µl cold 1 mM ATP, 134 µl water. Samples were incubated for 30 min (37 °C) then apyrase (2 µl, 80 mU) was added, followed by a further incubation of 30 min (37 °C). Apyrase digestion removes ^{32}P-ATP from the origin of the TLC plates and cleans up the background radioactivity.

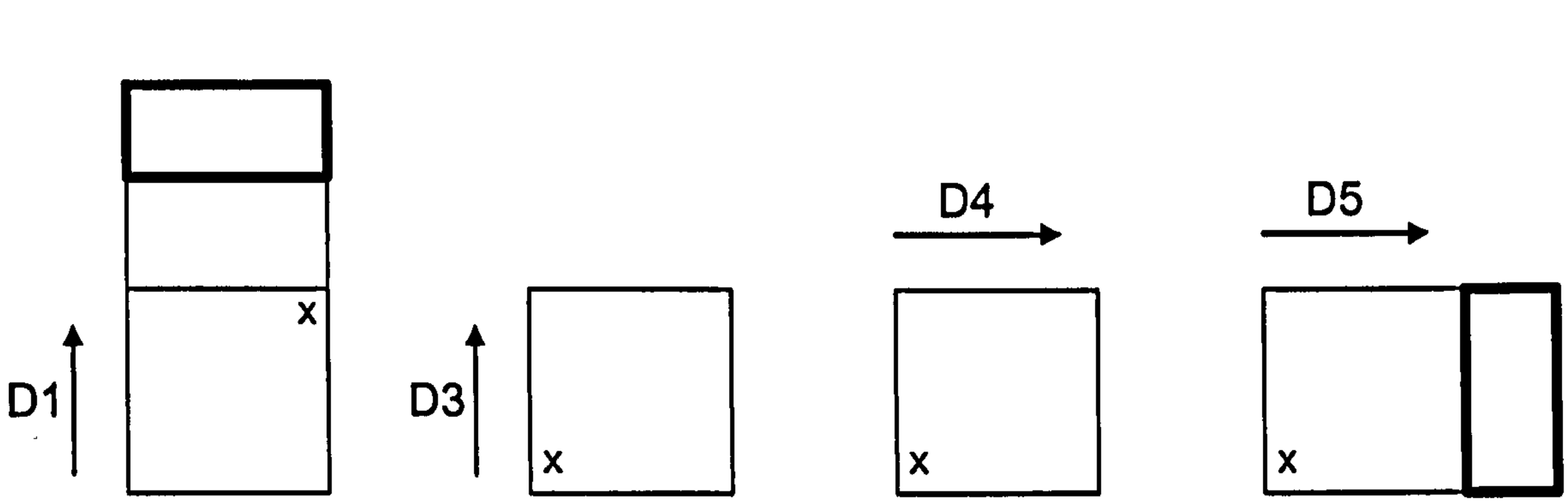
3.2.3.3 Chromatography of normal nucleotides

One aliquot was diluted to 100 µl with tris EDTA, and spotted (2 µl) onto polyethyleneimine (PEI)-cellulose thin layer chromatography (TLC) plates (Merck Eurolab Ltd., Poole, Dorset UK), and developed in 0.12 M sodium phosphate, pH 6.8 for approximately 2 hours. The plate was dried, wrapped in Saran wrap and scanned using a phosphorimager (Molecular Dynamics, Sevenoaks, Kent, UK).

3.2.3.4 Chromatography of adduct samples

The remaining aliquots were spotted onto a PEI-cellulose TLC plate (Camlab, Over, Cambs., UK). Unadducted nucleotides and other radioactive contaminants such as

[^{32}P]phosphate and unused [γ-^{32}P]ATP were removed from the plates onto Whatman No. 17 filter paper using solvent D1 (2.3 M sodium phosphate, pH 5.8) overnight (17 h). Filter paper wicks were removed and plates washed in distilled water for 5 min to remove salt. Plates were developed in ascending direction in solvent D3 (8.5 M urea, 4.0 M lithium formate, pH 3.5, 30°C). The pH was adjusted with a 13 mM tris base wash, then the plates were developed in the 2nd dimension with solvent D4 (8.5 M urea, 0.8 M LiCl, 0.5 M Tris-HCl, pH 8.5, 30°C). Prior to development in D3 and D4, plates were pre-developed to the origin in water to encourage uniform migration of the solvent front. Finally plates were developed in solvent D5 (1.7 M Sodium phosphate, pH 6) overnight onto wicks, to remove residual background radioactivity. Origins were excised, the plates were dried, wrapped in Saran wrap and scanned using a phosphorimager.



Normals and excess [γ^{32}P]-ATP are washed onto the filter paperwick. Adducts stay at the origin

Figure 3.3. Chromatography of adduct samples

3.2.4 Fugene-6 mediated transfection of Ad293 cells with BPDE treated pSP189 plasmid DNA

Ad293 cells (grown at 37°C, 5% CO_2, 90% confluent) were split 1 in 10 (approximately 2×10^6 cells) and plated in 9 cm transfection plates in 10 ml Dulbecco's Minimum Eagle's medium (Life Technologies Ltd., Paisley, UK) (with glutamax 1 and pyridoxine, without glutamine and sodium pyruvate, 10% foetal calf serum) and grown for 68 h (37°C, 5% CO_2). Cells were 40-60% confluent prior to transfection. Three hours before transfection, used medium was aspirated and fresh medium added (10 ml). Fugene-6 (Roche Diagnostics Ltd, Lewes, East Sussex, UK) transfection reagent (15 µl) was slowly added to foetal calf serum free Dulbecco's Minimum Eagle's medium (500 µl). The tube was gently tapped to mix the contents. Plasmid DNA (10 µg) was added to the Fugene solution and incubated at room temperature for 20 min. The aliquot of DNA was carefully added dropwise to each transfection plate and swirled gently to mix. Plates were then returned to the incubator (37°C, 5% CO_2). Twenty-four hours after transfection, used media was aspirated and replaced with fresh media (10 ml). The plasmid was reclaimed from the Ad293 cells between 45-48 h after transfection as described in Chapter 2.

3.2.5 Digestion of pSP189 plasmid with Dpn1 restriction enzyme

Recovered plasmid was incubated with Dpn1 restriction enzyme (New England Biolabs (UK) Ltd., Hitchin, UK) (2 U, 37°C, 3 h) in NEBuffer 4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol {pH 7.9

at 25°C}) in a total volume of 50 µl. Restriction enzyme was removed using Micropure EZ enzyme removers (following manufacturer's protocol, which involved repeated microcentrifugation) (Millipore Corporation, Bedford, MA, USA). The DNA was precipitated as above (Section 3.2.1) and re-dissolved in 20 µl tris-EDTA buffer, pH 8.0 and stored at -20°C prior to transformation of electrocompetent *E.coli*, see Chapter 2. Treatment of plasmid with the Dpn1 restriction enzyme, after it has been recovered from human cells, will digest any plasmid molecules which have not been produced by replication. Dpn1 does this by recognising methylated adenosines in the sequence 5'-GA^TC-3' in DNA and catalysing a double stranded cut on the DNA strand 3' to the adenine. All plasmid molecules that have been replicated in the human cells will have lost their methylated adenosines, as only bacterial cells possess the appropriate methylating enzymes, and will therefore remain in covalently closed circular form.

3.3 Results

In order to assess the mutagenic effects of short and medium wavelength ultraviolet radiation (UVB, 302 nm and UVC, 254 nm) in combination with B[*a*]P, the shuttle vector plasmid pSP189 was modified by *in vitro* treatment with a single dose of BPDE before or after treatment with UVB (2.4 kJ/m^2) or UVC (0.6 kJ/m^2) radiation. Following replication in human adenovirus transformed kidney (Ad293) cells, recovered plasmid was used to transform MBM7070 indicator *E. coli*. Any plasmids containing a mutation in the *supF* gene grew as white colonies whilst non-mutant wild type plasmids grew as blue colonies.

3.3.1 Part A: Treatment with BPDE followed by UVB or UVC radiation

This work was carried out as part of an ongoing collaboration between this laboratory and Dr. Michael N. Routledge. The experimental procedures which produced the results in Part A were undertaken jointly by the candidate and Dr. Routledge, whilst he was previously employed at De Montfort University, Leicester. The candidate performed tissue culture, plasmid preparations, transfections, transformations, sequencing analysis and general methods related to the project. Dr. Routledge treated and postlabelled plasmid DNA, transformed *E.coli* and picked / counted colonies. This data is included in the publication: Routledge, M.N., McLuckie, K.I.E., *et al.*, (2001) Presence of benzo[*a*]pyrene diol epoxide adducts in target DNA leads to an increase in UV-induced DNA single strand breaks and *supF* gene mutations. *Carcinogenesis*, **22**, 1231-1238. The follow up experiments in Part B were performed by the candidate, with postlabelling performed by Margaret Gaskell.

3.3.1.1 BPDE DNA adduct quantification by ^{32}P-postlabelling

Aliquots of the BPDE modified plasmid were ^{32}P-postlabelled before further treatment with UVB or UVC radiation. The DNA damage level was 2.2 adducts per 10^4 nucleotides.

3.3.1.2 Mutation frequency in *supF* gene

The spontaneous mutation frequency of plasmid treated with water only (0.2×10^{-4}) was of a comparable value to those previously reported in Ad293 cells. Bigger, *et al.* (1990 and 1992) reported spontaneous mutation frequencies of 0.5 and 0.2×10^{-4}, whilst Boldt, *et al.* (1991) reported 0.8×10^{-4}. Treatment with BPDE resulted in a 40-fold increase in mutation frequency compared to solvent only control (Table 3.1).

Table 3.1. Mutation frequency induced by the various treatments of pSP189 plasmid

Treatment	Colonies Screened	Number of Mutants	Mutation Frequency[a]
Solvent control[b]	216000	6	0.3
BPDE[c]	94000	112	12
UVB	3000	134	446
BPDE + UVB	3000	350	1167
UVC	18000	47	26
BPDE +UVC	13000	256	197

[a] Mutation frequency per 10^4 colonies

[b] Mutation frequency of plasmid dissolved in water only was 0.2×10^{-4}

[c] BPDE treated plasmid contained 2.2 adducts per 10^4 nucleotides

The UV treatments alone also gave a marked increase in mutation frequency over control, UVB inducing an almost 1500-fold increase whilst UVC induced a 86-fold increase in mutation frequency. When BPDE treatment was combined with UV treatment, an even larger increase in mutation frequency was observed. BPDE

followed by UVB induces a 3890-fold increase in mutation frequency over control. This is 97-fold greater than BPDE alone and 2.6-fold greater than UVB alone. BPDE followed by UVC induces a 656-fold increase in mutation frequency over control. This is 16-fold greater than BPDE alone and 7.6-fold greater than UVC alone.

3.3.1.3 Mutation types found in *supF* gene

Due to the low spontaneous mutation frequency induced by solvent control plasmid (acetone), a mutation spectrum has been compiled from previously published *supF* studies using Ad293 cells (Bigger, *et al.*, 1990 and 1992; Boldt, *et al.*, 1991; and Juedes and Wogan, 1996). The control plasmid in these reports were treated with ethanol, potassium nitrate, sodium succinate, or tris-HCL. The majority of spontaneous mutations are in the form of single base substitutions (64% of all mutations, Table 3.2) followed by multiple mutations (21%), along with a small amount of frameshift mutations, in the form of deletions. The spontaneous mutations seen in this study were three single base substitutions (two GC→TA transversions and one GC→AT transition), two multiple base deletions, a double base insertion and a nine base insertion.

All treatments induced base substitutions as the major mutations (Table 3.2). All of the mutations induced by treatment with BPDE were in the form of single base substitutions. UVB treatment alone induced 97% of all mutations in the form of base substitutions: 82% in the form of single base substitutions, 1.5% as tandem (two adjacent bases substituted) and 4.4% as multiple base substitutions (two or more substitutions at non adjacent sites). The other 3% of mutations corresponds to two single base deletions of a C at position 108 and a C at position 172.

Table 3.2. Types of sequence alterations in *supF* gene of pSP189 plasmids treated with benzo[*a*]pyrene diol epoxide and /or UV radiation replicated in Ad293 cells

Mutations	Number of plasmids with mutations (%)					
	Control[a]	BPDE	UVB	BPDE + UVB	UVC	BPDE + UVC
Base substitutions	**63** (86)	**46** (100)	**66** (97)	**78** (100)	**34** (100)	**66** (96)
Single	47 (64)	46 (100)	56 (82)	55 (71)	31 (91)	49 (71)
Tandem	1 (9.1)	0 (0)	7 (1.5)	6 (7.7)	3 (8.8)	5 (7.2)
Multiple	15 (21)	0 (0)	3 (4.4)	17 (22)	0 (0)	12 (17)
Frameshifts	**10** (14)	**0** (0)	**2** (2.9)	**0** (0)	**0** (0)	**3** (4.3)
Single base deletion	4 (5.5)	0 (0)	2 (2.9)	0 (0)	0 (0)	0 (0)
>2 bases deletion	6 (8.2)	0 (0)	0 (0)	0 (0)	0 (0)	3 (4.3)
Single base insertion	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
>2 bases insertion	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total plasmids sequenced	**73** (100)	**46** (100)	**68** (100)	**78** (100)	**34** (100)	**69** (100)

[a] Solvent controls adapted from Bigger, et al., 1990 and 1992; Boldt, *et al.*, 1991 and Juedes and Wogan, 1991.

BPDE followed by UVB treatment induced only base substitutions, 71% of which were single base substitutions, 7% tandem and 22% were multiple base substitutions. UVC alone also induced only base substitutions (91% single, and 9% tandem). Whereas BPDE followed by UVC also induced multiple base substitutions (17%), along with 71% as single base substitutions and 7% as tandem substitutions and large deletions (4.3%).

As can be seen in Tables 3.3 and 3.4 there is a distinct difference in the pattern of base substitution types seen after the various treatments and those generated from control plasmids. In the control spectrum the major substitution is the GC→TA transversion (51% of all single base substitutions), occurring over 2-fold more frequently than GC→CG transversions (20%) and GC→AT transitions (18%). AT→TA transversions (6%) and AT→CG transversions (4%) were seen but no AT→GC transitions. In the control mutation spectrum of multiple mutations there is a large preference for GC→AT transitions (67% of all multiple mutations), with a smaller

proportion of GC→TA (19%), GC→CG (7%), AT→TA, and AT→CG (4% each) transversions.

Table 3.3. Types of single and tandem base substitution mutations in *supF* gene of pSP189 plasmids treated with benzo[*a*]pyene diol epoxide and /or UV radiation replicated in Ad293 cells

Mutations	Number of plasmids with mutations (%)					
	Control[a]	BPDE	UVB	BPDE + UVB	UVC	BPDE + UVC
Transversions	**40** (82)	**40** (87)	**12** (17)	**15** (22)	**7** (19)	**21** (36)
GC→TA	25 (51)	30 (65)	5 (7)	10 (15)	3 (8)	14 (24)
GC→CG	10 (20)	10 (22)	0 (0)	1 (1)	3 (8)	6 (10)
AT→TA	3 (6)	0 (0)	3 (4.3)	4 (6.0)	1 (2.7)	1 (1.7)
AT→CG	2 (4)	0 (0)	4 (5.7)	0 (0.0)	0 (0)	0 (0.0)
Transitions	**9** (18)	**6** (13)	**58** (83)	**52** (78)	**30** (81)	**38** (64)
GC→AT	9 (18)	5 (11)	58 (83)	50 (75)	29 (78)	35 (59)
AT→GC	0 (0)	1 (2.2)	0 (0)	2 (3)	1 (2.7)	3 (5)
Total single base substitutions	**49** (100)	**46** (100)	**70** (100)	**67** (100)	**37** (100)	**59** (100)

[a] Solvent controls adapted from Bigger, *et al.*, 1990 and 1992; Boldt, *et al.*, 1991 and Juedes and Wogan, 1991.

In the mutation spectra induced by treatment with BPDE the major substitution is the GC→TA transversion (65% of all single base substitutions), occurring almost 3-fold more frequently than GC→CG transversions (22%) and 5-fold more frequently than transversions (13% of all substitutions, 11% as GC→AT, and 2% as AT→GC). UVB irradiation alone induced mainly transitions, with 83% of all single and tandem base substitutions being GC→AT transitions. The remainder were GC→TA (7%), AT→TA (4%) and AT→GC (6%) transversions. In the UVB mutation spectrum of multiple mutations there were three AT→GC transitions, two GC→AT transitions and a single GC→TA transversion. When plasmid was treated with BPDE then irradiated with UVB the induced mutations resembled those of UVB treatment rather than BPDE treatment, with a large amount (78%) of transitions (75% as GC→AT, and 3% as AT→GC), and relatively few transversions (22% overall). There was a marked increase in the number of plasmids with multiple mutations (22% of all plasmids sequenced). These were also predominantly transitions (64%), with the

major mutation being the GC→AT substitution (56%), rather than transversions (36%).

Table 3.4. Types of multiple base substitution mutations in *supF* gene of pSP189 plasmids treated with benzo[*a*]pyrene diol epoxide and /or UV radiation replicated in Ad293 cells

Mutations	Number of plasmids with mutations (%)			
	Control[a]	UVB	BPDE + UVB	BPDE + UVC
Transversions	**9** (33)	**1** (17)	**18** (36)	**13** (39)
GC→TA	5 (19)	1 (17)	8 (16)	6 (18)
GC→CG	2 (7.4)	0 (0)	6 (12)	2 (6)
AT→TA	1 (4)	0 (0)	1 (2)	5 (15)
AT→CG	1 (4)	0 (0)	3 (6)	0 (0.0)
Transitions	**18** (67)	**5** (83)	**32** (64)	**20** (61)
GC→AT	18 (67)	2 (33)	28 (56)	16 (48)
AT→GC	0 (0)	3 (50)	4 (8)	4 (12)
Total multiple base substitutions	**27** (100)	**6** (100)	**50** (100)	**33** (100)

[a] Solvent controls adapted from Bigger, *et al.*, 1990 and 1992; Boldt, *et al.*, 1991 and Juedes and Wogan, 1991.

UVC treatment induces a similar mutation profile to that seen with UVB treatment. The majority of single base and tandem substitutions are in the form of GC→AT transitions (78%) and are almost 10-fold more numerous than the next most frequent transversion mutations (GC→TA, and GC→CG, 8% each). No multiple mutations were seen for UVC alone, but when plasmid was irradiated with UVC after treatment with BPDE 17% of the sequenced plasmids contained two or more mutations. The major multiple substitution was the GC→AT transition (48% of all substitutions), which was 2.7 fold more frequent than GC→TA transversions and 3.2 or 4-fold more frequent than AT→TA transversions, and AT→GC transitions, respectively. When plasmid was treated with BPDE then irradiated with UVC the induced mutations most resembled those of UVC treatment rather than BPDE treatment, as was seen for BPDE followed by UVB irradiation.

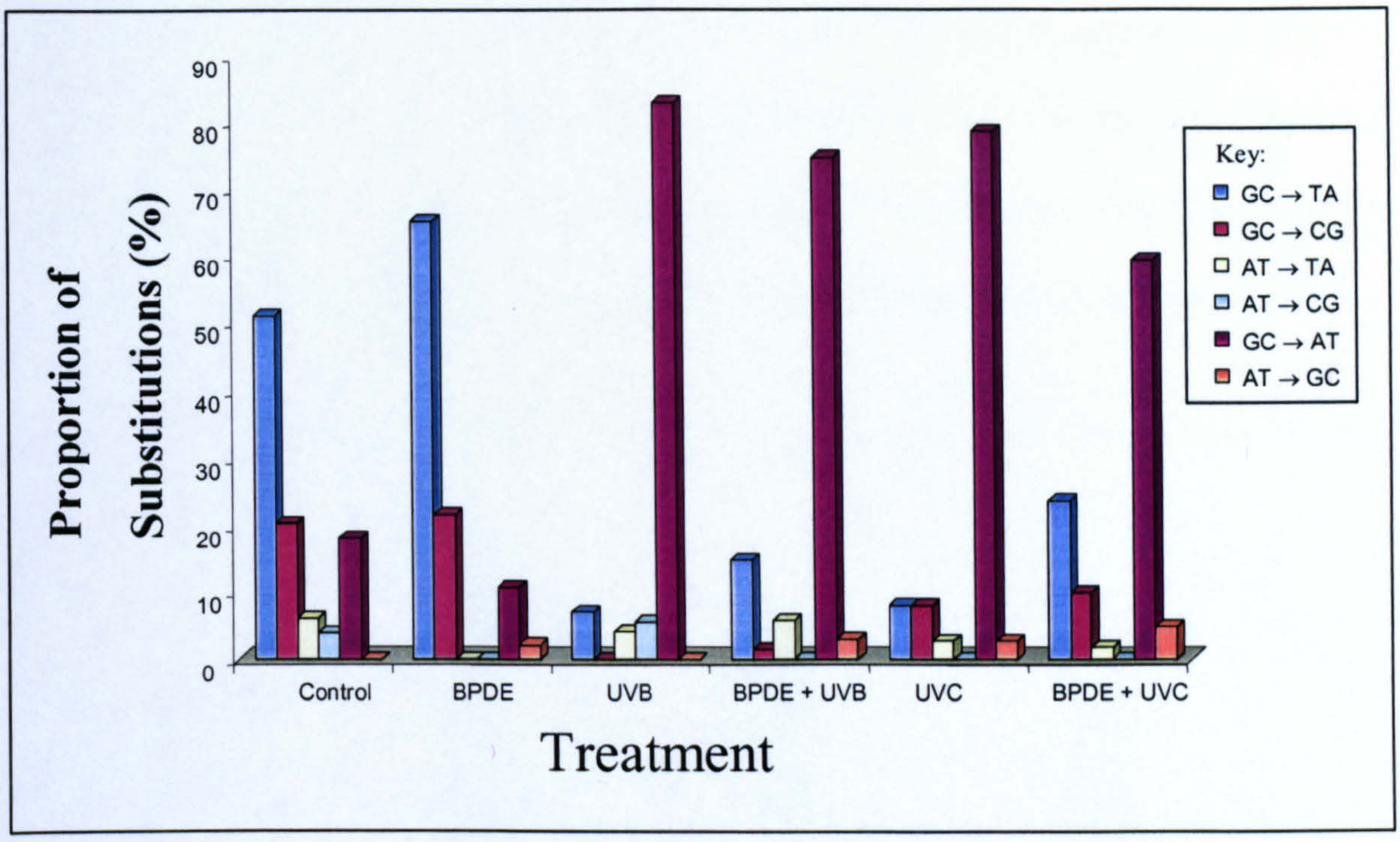


Figure 3.4 Bar chart illustrating the proportion of the different single base and tandem substitution mutations seen in control mutants and in those induced by treatment with BPDE, UVB (303 nm), BPDE followed by UVB, UVC (254 nm) and BPDE followed by UVC. Control mutations taken from Bigger, *et al.,* (1990 and 1992), Boldt, *et al.,* (1991) and, Jeudes and Wogan (1996).

Figure 3.4 illustrates the comparison of the different proportions of base substitution mutations induced in the *supF* gene, by solvent control and by the various treatments. It can be observed that treatment with BPDE induces a spectrum of mutations which is similar to that seen in published control mutation spectra, rather than with UV treatment or UV plus BPDE treatment. All treatments involving UVB or UVC irradiation had the GC→AT transition as the most frequent mutation. Treatment with BPDE as well as UV resulted in a slight increase in transversions, especially GC→TA, although these were still in the minority. Solvent control mutations, as well as those induced by BPDE alone, were predominantly transversions. When multiple

mutations are expressed in this way (Figure 3.5), the GC→AT transition is, again, the major mutation for all treatments (that induced multiple mutations) including published solvent controls. The relative amount of transversions varied between the three treatments, whilst always being below around 20% per mutation type.

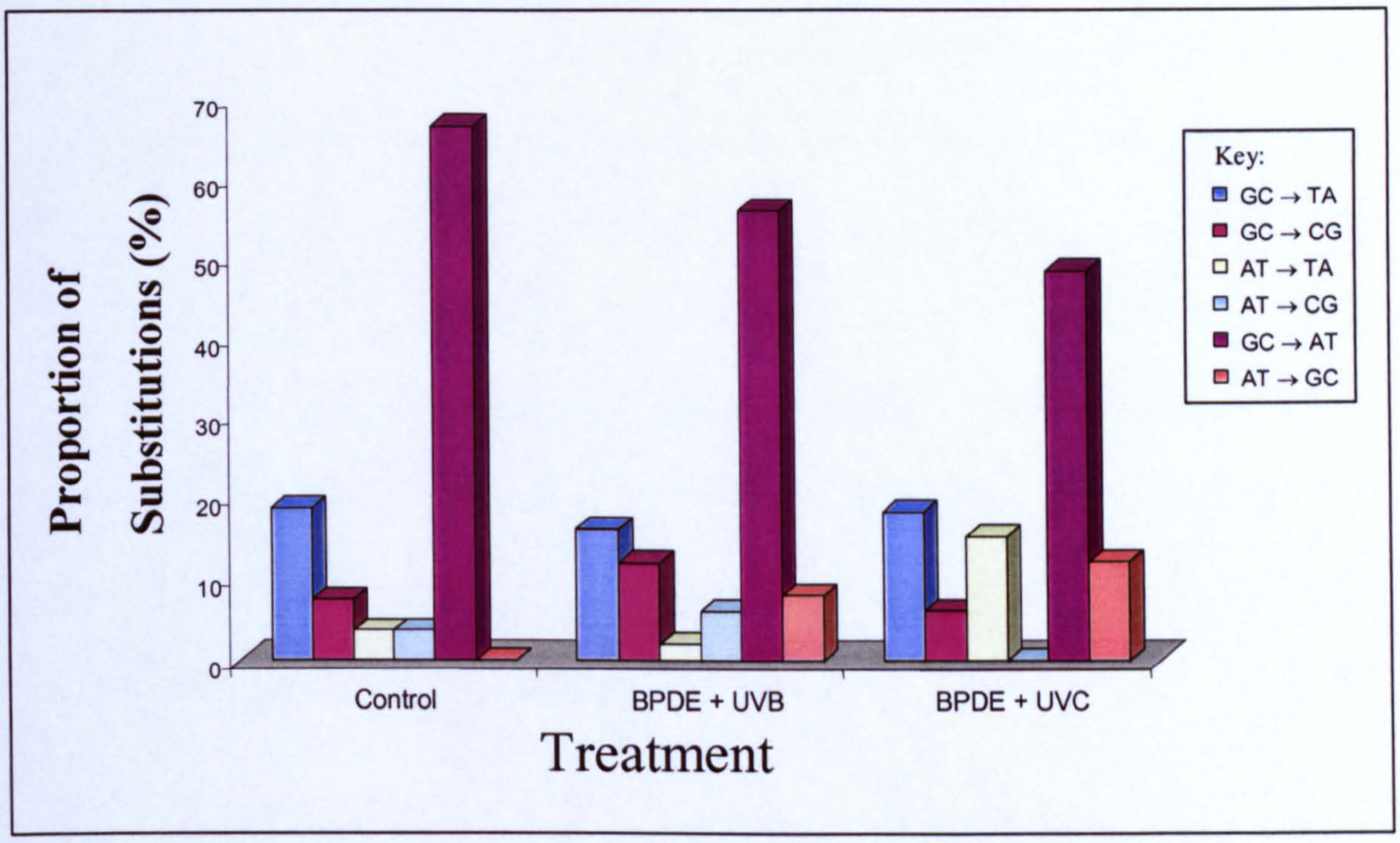


Figure 3.5 Bar chart illustrating the proportion of the different multiple base substitution mutations seen in control mutants and in those induced by treatment with BPDE followed by UVB, and BPDE followed by UVC. Control mutations taken from Bigger, *et al.*, (1990 and 1992), Boldt, *et al.*, (1991) and, Jeudes and Wogan (1996).

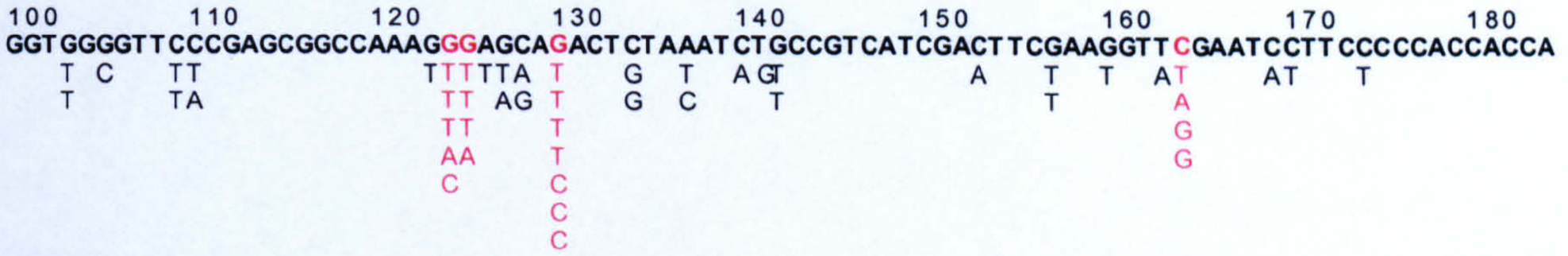
3.3.1.4 Mutation spectra in *supF* gene

The distribution of single base and tandem substitution mutations within the *supF* gene for plasmids dosed with the various BPDE, UV irradiation or control treatments is shown in Figure 3.6. When these spectra are compared using the hyperg program

(Cariello, *et al.*, 1994), treated spectra are found to be significantly different from control (p (same) $\leq$ 0.05). Mutations are not distributed randomly, but concentrated at one or more sites, known as hotspots. In the mutation spectrum induced in solvent treated plasmids there are four hotspots at positions 123, 124, 129, and 163, all of which are at GC basepairs. Treatment of plasmid with BPDE induces hotspots at positions 110, 115 and 175 of the *supF* gene, all of which are also GC basepairs. Irradiation with UVB induces hotspots at six positions (108, 122, 155, 156, 159, and 172, all at GC basepairs). BPDE treatment followed by irradiation with UVB induces mutation hotspots in the *supF* gene preferentially at the GC basepairs of positions 155, 156, 159, and 163. Treatment of plasmid with UVC (with and without BPDE) also induces hotspots at GC basepairs in the *supF* gene. These are at positions 104, 123, 156, 159, and 172 for UVC only, and at positions 110, 155, 163, 172, and 175 for BPDE treatment followed by UVC irradiation.

The distribution of multiple base substitution mutations within the *supF* gene for plasmids dosed with the various BPDE followed by UV irradiation or control treatments is shown in Figure 3.7. In the solvent control multiple spectrum there are three hotspots at positions 113, 129 and 150, all of which are at GC basepairs. Treatment with BPDE followed by UVB irradiation did not induce any mutation sites which were significantly more numerous than for a random distribution. Treatment with BPDE followed by UVC irradiation, however, did induce mutation hotspots at positions 156 and 172. All hotspot sites in both single and multiple base substitution mutation spectra were at sites of GC basepairs.

Control Mutations



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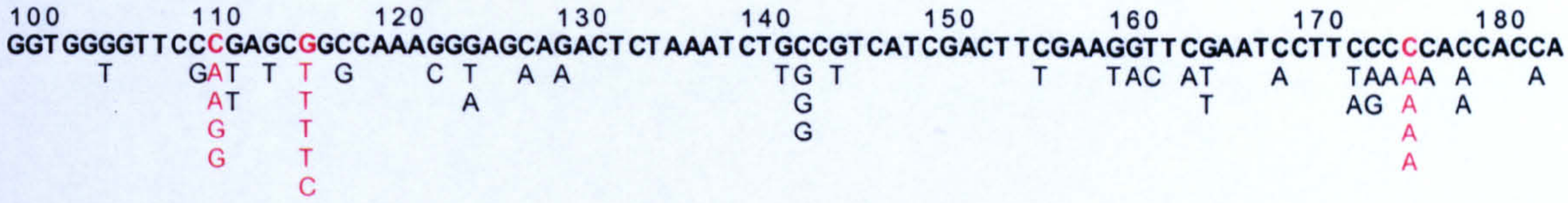

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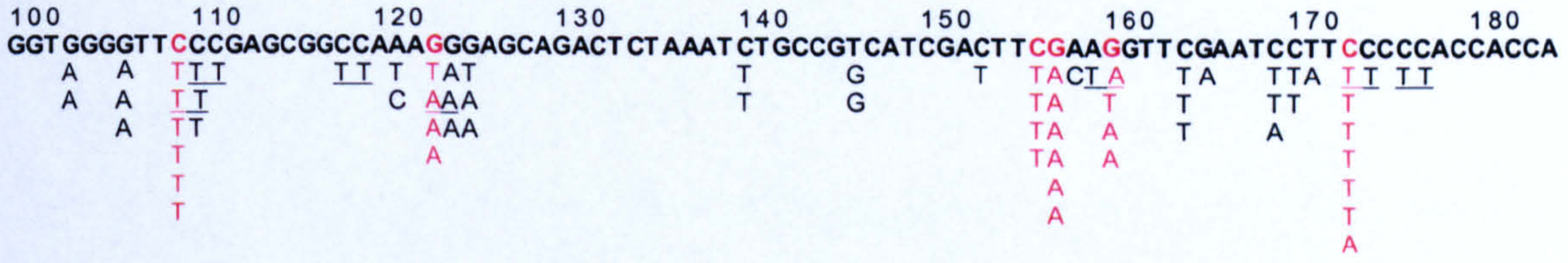
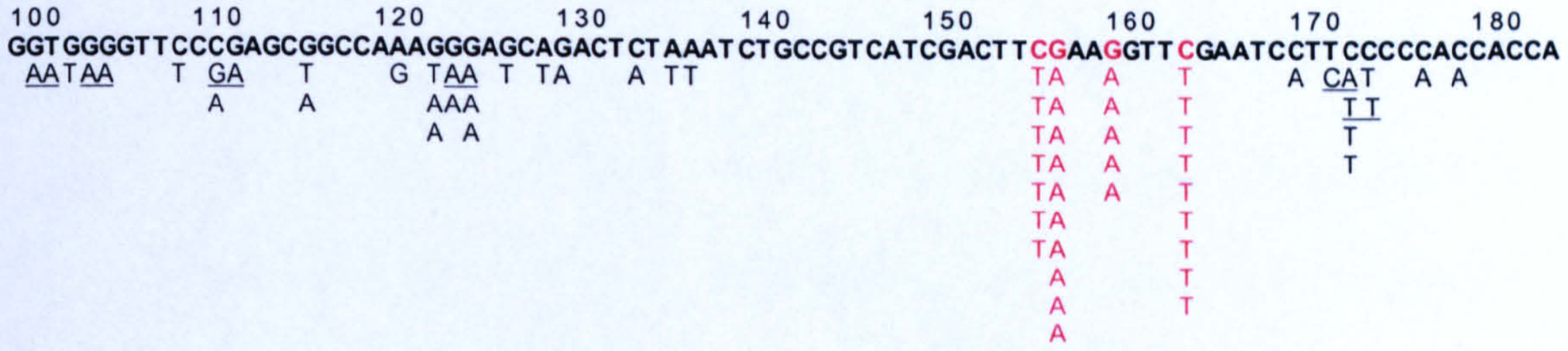
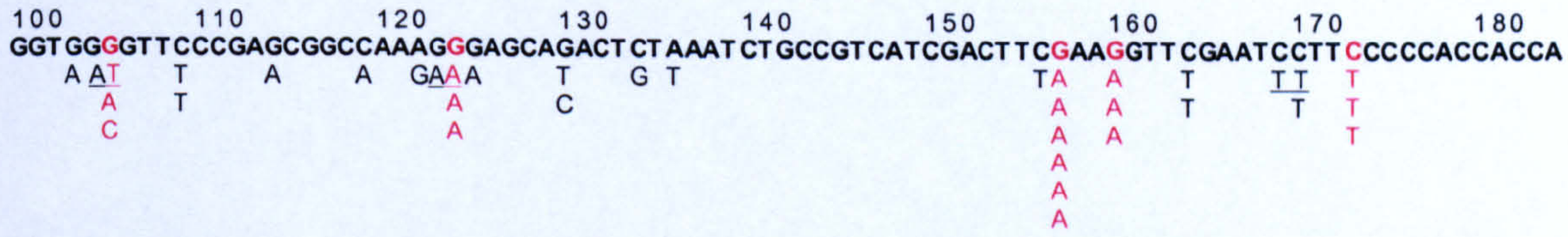


BPDE + UVB



UVC



BPDE + UVC

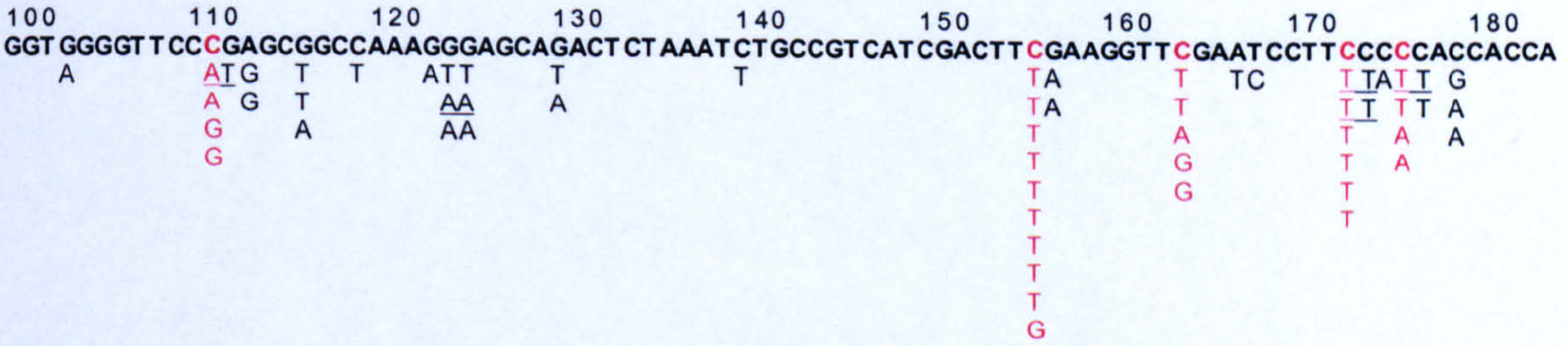
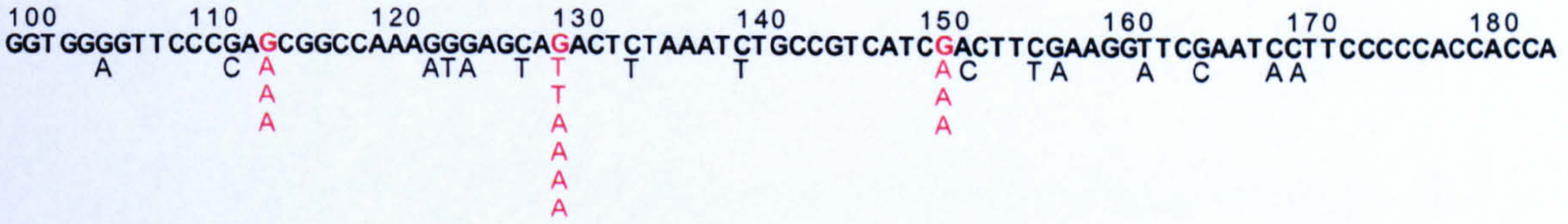
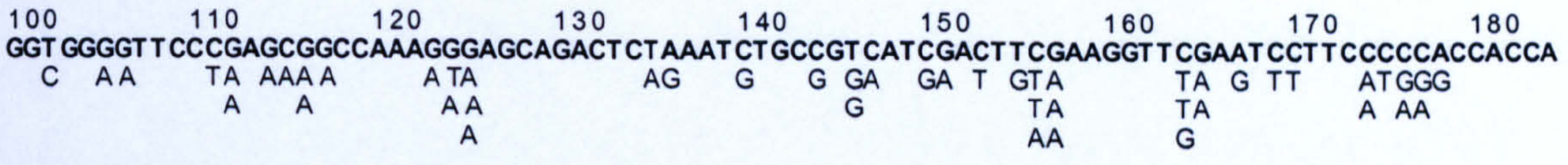


Figure 3.6 Mutation spectra depicting single base and tandem substitutions induced in control plasmids and after the various treatments. Control spectrum taken from Bigger, *et al.,* (1990 and 1992), Boldt, *et al.,* (1991) and, Jeudes and Wogan (1996). Hotspots are shown in red.

Control Mutations



BPDE + UVB



BPDE + UVC

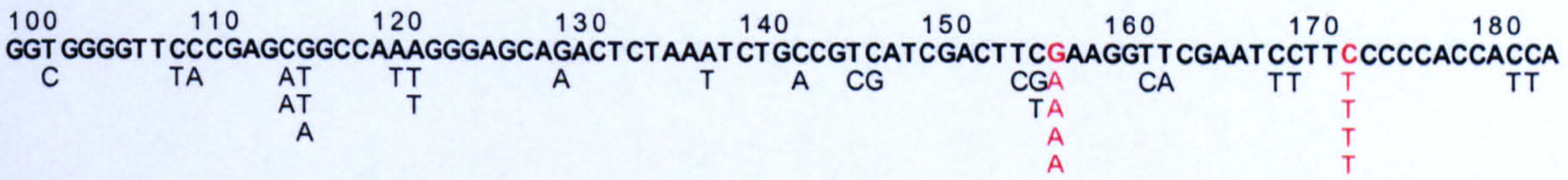


Figure 3.7 Mutation spectra depicting multiple base substitutions induced in control plasmids and after the various treatments. Control spectrum taken from Bigger, *et al.*, (1990 and 1992), Boldt, *et al.*, (1991) and, Jeudes and Wogan (1996). Hotspots are shown in red.

Some sites in the *supF* gene seemed to appear as hotspots in more than one spectrum. Position 110 was a hotspot after BPDE treatment and BPDE with UVC treatment. Position 129 appeared in both control spectra: in the single and tandem substitutions spectrum and in the multiple substitutions spectrum. Position 155 was a hotspot with UVB, BPDE and UVB and with BPDE and UVC. Positions 156 and 159 were hotspots with UVB, BPDE plus UVB and with UVC treatment, whilst position 156 also had a hotspot in the BPDE plus UVC multiple spectrum. There was a hotspot at position 172 in UVB, UVC, and in both the BPDE and UVC single and multiple spectra. Position 175 appeared as a hotspot after treatments of BPDE alone and BPDE plus UVC (single base substitution spectrum).

3.3 2 Part B: Treatment with UVB or UVC radiation followed by BPDE

Treatment with UVB and UVC followed by BPDE was investigated to see whether the order in which DNA damage was induced had any affect on its mutagenicity. This experiment was designed to determine whether the mutation frequencies observed after the binary treatments of BPDE and UV were due to the chemical activation of BPDE adducts by UV, or were due to some other mechanism. This experiment, including DNA treatment, ^{32}P-postlabelling and mutation analysis, was carried out at a later time than that described in Section 3.2.1.

3.3.2.1 BPDE DNA adduct quantification by ^{32}P-postlabelling

The magnitude of DNA damage induced by BPDE was quantified using ^{32}P-postlabelling.

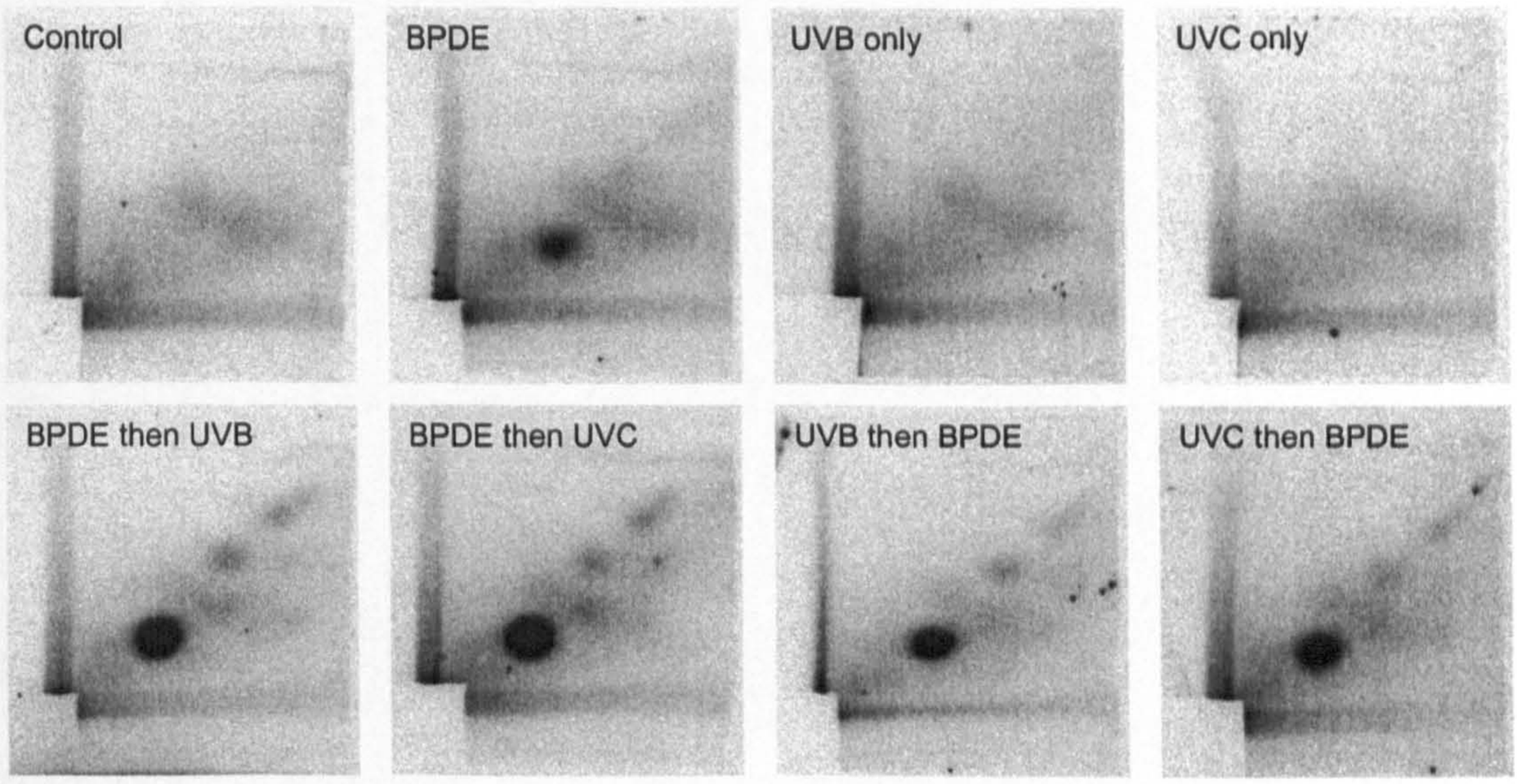


Figure 3.8 Examples of the radioactive thin layer chromatogams (TLC), showing the patterns of adducts induced by the different treatments. (Visualisation and quantification analysis by phosphorimaging)

Samples for all treatments were analysed, including those not involving BPDE (Figure 3.8). Plasmid was also treated as in part A, so that a comparison of adduct yields could be made.

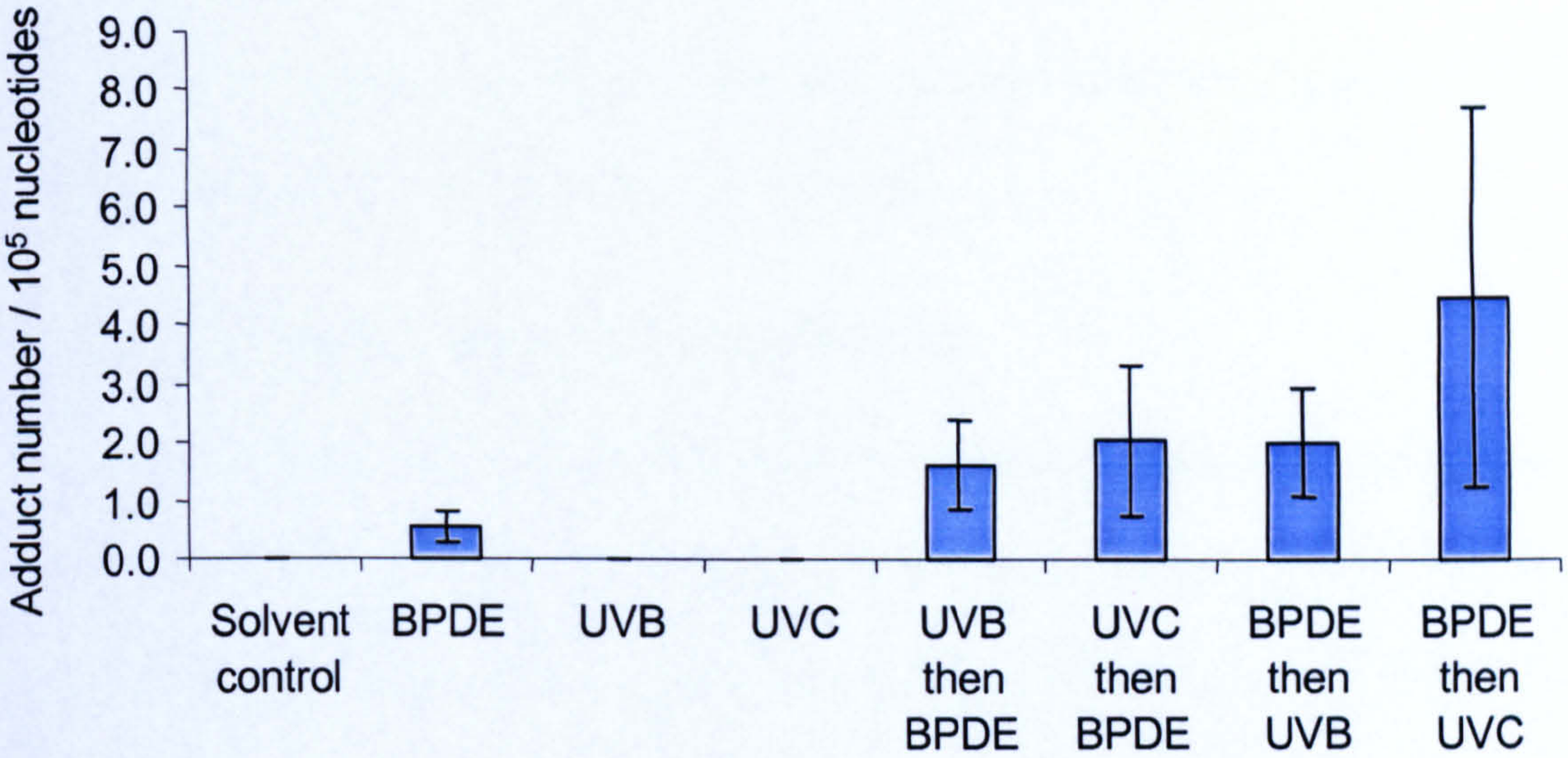


Figure 3.9 DNA adduct levels for plasmid treated with BPDE, UVB, UVC and solvent control. Error bars represent standard error.

Control, UVB and UVC treatments all showed a lack of visible DNA adduct spots on the TLC plates. The bar chart in Figure 3.9 illustrates the total amounts of adducts after the different treatments. The adduct levels observed in this experiment were an order of magnitude lower than in part A, since a standard was not available to correct for experimental variation, this may be due to variables in the ^{32}P-postlabelling assay (e.g. enzyme efficiencies). It can be seen that, rather surprisingly, the DNA adduct level increases when BPDE is combined with UV irradiation, irrespective of the order of treatment. BPDE treatment alone yielded 0.5 adducts per 10^5 nucleotides whilst combined treatments yielded 1.6 or 2 adducts per 10^5 nucleotides when UV was administered first (Table 3.5) and 2 or 4.5 adducts per 10^5 nucleotides when BPDE

was administered first (Figure 3.9). Whilst, the mean level of adducts for treatment with BPDE then UVC is not significantly higher than when UVC is treated first, this is most likely due to the differences between postlabelling experiments. Data from these individual experiments does show an actual increase between the different treatments (Appendix 1). A HPLC method for analysis of ^{32}P-postllabelled BPDE adducts is being developed in our laboratory so that further investigation of BPDE and UV interactions with DNA can be performed.

3.3.2.2 Mutation frequency in *supF* gene

The mutation frequency of control plasmid, treated with solvent only, was around 6-fold higher in this experiment than observed previously (Section 3.3.1.2). Treatment with BPDE only resulted in an 8-fold increase in mutation frequency over control (Table 3.5).

Table 3.5. Mutation frequency induced by the various treatments of pSP189 plasmid

Treatment	Mutation Frequency[a]	Adduct Number (± S.E)
Control[c]	1.7	0 ± 0
BPDE	14	0.5 ± 0.26
UVB	35	0 ± 0
UVC	47	0 ± 0
UVB then BPDE	56	1.6 ± 0.76
UVC then BPDE	101	2 ± 1.3

[a] Mutation frequency per 10^4 colonies
[b] Adduct number per 10^6 nucleotides
[c] Mutation frequency of plasmid dissolved in water only was 0.2×10^{-4}

Treatment with UVB only resulted in a 20-fold increase in mutation frequency over control, but was an order of magnitude lower than in the previous experiment. Treatment with UVB followed by BPDE resulted in a mutation frequency increase of 33-fold over control. This was 20-fold less than when plasmid was treated with

BPDE followed by UVB (Section 3.3.1.2). In this experiment the mutation frequency when plasmid was treated with UVC only was slightly higher than in the previous experiment (47×10^{-4} versus 26×10^{-4}, respectively), this was almost 28-fold greater than control. When plasmid was treated with BPDE as well, the mutation frequency increased by a factor of two. This is also lower than in the previous experiment where treatment with BPDE followed by UVC was 7.6-fold more mutagenic than UVC treatment alone.

3.3.2.3 Mutation types found in *supF* gene

As in part A, due to the low spontaneous mutation frequency induced by control plasmid, a mutation spectrum has been compiled from previously published *supF* studies using Ad293 cells (Bigger, *et al.*, 1990 and 1992; Boldt, *et al.*, 1991; and Juedes and Wogan, 1996). The solvent control is therefore the same as in part A.

Table 3.6. Types of sequence alterations in *supF* gene of pSP189 plasmids treated with UVC radiation followed by benzo[*a*]pyrene diol epoxide replicated in Ad293 cells

Mutations	Number of plasmids with mutations (%)				
	Control[a]	BPDE[b]	UVC[b]	BPDE then UVC[b]	UVC then BPDE
Base substitutions	**63** (86)	**46** (100)	**34** (100)	**66** (96)	**97** (91)
Single	47 (64)	46 (100)	31 (91)	49 (71)	84 (79)
Tandem	1 (9.1)	0 (0)	3 (9)	5 (7)	4 (4)
Multiple	15 (21)	0 (0)	0 (0)	12 (17)	9 (8.4)
Frameshifts	**10** (14)	**0** (0)	**0** (0)	**3** (4)	**10** (9.3)
Single base deletion	4 (5.5)	0 (0)	0 (0)	0 (0)	4 (3.7)
>2 bases deletion	6 (8.2)	0 (0)	0 (0)	3 (4)	4 (3.7)
Single base insertion	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.9)
>2 bases insertion	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.9)
Total plasmids sequenced	**73** (100)	**46** (100)	**34** (100)	**69** (100)	**107** (100)

[a] Solvent controls adapted from Bigger, *et al.*, 1990 and 1992; Boldt, *et al.*, 1991 and Juedes and Wogan, 1991.

[b] Mutation data from Part A

Mutant colonies were picked from recovered plasmid treated with UVC radiation followed by BPDE to produce a mutation spectrum. A comparison with the BPDE alone, UVC alone, and BPDE followed by UVC (single and multiple) mutation spectra could then be made (from Part A). Table 3.6 shows the types of sequence alterations found in plasmids treated with UVC followed by BPDE compared to solvent control, BPDE alone, UVC alone and the reverse treatment, BPDE followed by UVC. As can be seen from Table 3.6, the majority of mutations induced by UVC followed by BPDE were in the form of base substitutions (91% of all mutant plasmids) with only a few deletions and insertions (8% and 2%, respectively). The number of tandem and multiple base substitutions has decreased slightly (cf. BPDE then UVC, 4% and 8.4% versus 7% and 17%, respectively). The percentage of deletions has doubled, and insertions are now seen (2% of all mutations), when they were absent following treatment with BPDE followed by UVC.

The types of base substitution mutation induced by plasmid treatment with UVC followed by BPDE can be seen in Table 3.7. The relative amounts of the different transversion and transition mutations do not seem to change significantly when the plasmid was treated with UVC followed by BPDE compared to BPDE followed by UVC. The percentage of transversions decreases when UVC is applied before BPDE treatment (36% to 29%) of which the majority are still GC→TA transversions (21%) with 5% being GC→CG and 3.3% AT→TA transversions. The amount of transitions has increased slightly from 64% when BPDE was applied first to 71% when UVC was applied first. Of these the majority are GC→AT transitions (63%), with a few AT→GC transitions.

Table 3.7. Types of single and tandem base substitution mutations in *supF* gene of pSP189 plasmids treated with UVC radiation followed by benzo[*a*]pyrene diol epoxide replicated in Ad293 cells

Mutations	Number of plasmids with mutations (%)				
	Control[a]	BPDE[b]	UVC[b]	BPDE then UVC[b]	UVC then BPDE
Transversions	**40 (82)**	**40 (87)**	**7 (19)**	**21 (36)**	**27 (29)**
GC→TA	25 (51)	30 (65)	3 (8)	14 (24)	19 (21)
GC→CG	10 (20)	10 (22)	3 (8)	6 (10)	5 (5)
AT→TA	3 (6)	0 (0)	1 (2.7)	1 (1.7)	3 (3.3)
AT→CG	2 (4)	0 (0)	0 (0)	0 (0.0)	0 (0.0)
Transitions	**9 (18)**	**6 (13)**	**30 (81)**	**38 (64)**	**65 (71)**
GC→AT	9 (18)	5 (11)	29 (78)	35 (59)	58 (63)
AT→GC	0 (0)	1 (2.2)	1 (2.7)	3 (5)	7 (8)
Total single base substitutions	**49 (100)**	**46 (100)**	**37 (100)**	**59 (100)**	**92 (100)**

[a] Solvent controls adapted from Bigger, *et al.*, 1990 and 1992; Boldt, *et al.*, 1991 and Juedes and Wogan, 1991.
[b] Mutation data from Part A

There is a more noticeable difference in the types of mutations seen in the multiple mutation spectra induced by UVC treatment before and after BPDE treatment (Table 3.8).

Table 3.8. Types of multiple base substitution mutations in *supF* gene of pSP189 plasmids treated with benzo[*a*]pyrene diol epoxide and UVC radiation replicated in Ad293 cells

Mutations	Number of plasmids with mutations (%)		
	Control[a]	BPDE then UVC[b]	UVC then BPDE
Transversions	**9 (33)**	**13 (39)**	**20 (67)**
GC→TA	5 (19)	6 (18)	8 (27)
GC→CG	2 (7.4)	2 (6)	6 (20)
AT→TA	1 (4)	5 (15)	5 (17)
AT→CG	1 (4)	0 (0.0)	1 (3.3)
Transitions	**18 (67)**	**20 (61)**	**10 (33)**
GC→AT	18 (67)	16 (48)	9 (30)
AT→GC	0 (0)	4 (12)	1 (3)
Total multiple base substitutions	**27 (100)**	**33 (100)**	**30 (100)**

[a] Solvent controls adapted from Bigger, *et al.*, 1990 and 1992; Boldt, *et al.*, 1991 and Juedes and Wogan, 1991.
[b] Mutation data from Part A

BPDE treatment followed by UVC induces mainly transitions (48% as GC→AT, 12% as AT→GC, 61% overall) with a smaller amount of transversions (18% as GC→TA, 6% as GC→CG, 15% as AT→TA, 39% overall). UVC treatment followed by BPDE

treatment, on the other hand, predominantly induces transversions. 67% of all multiple mutations were transversions, 27% as GC→TA, 20% as GC→CG, 17% as AT→TA and 3% as AT→CG, the majority of the transitions were GC→AT (30%) along with a single AT→GC transition. The different proportions of single and tandem base substitutions for solvent control, BPDE only, UVC only, BPDE followed by UVC and UVC followed by BPDE is illustrated graphically in Figure 3.10. The overall profile of mutation types looks quite similar whether plasmid DNA was treated with BPDE first or UVC first. Both complex plasmid treatments had more in common with the mutation profile induced by UVC treatment rather than with BPDE treatment or as a result of treatment with just the control solvent.

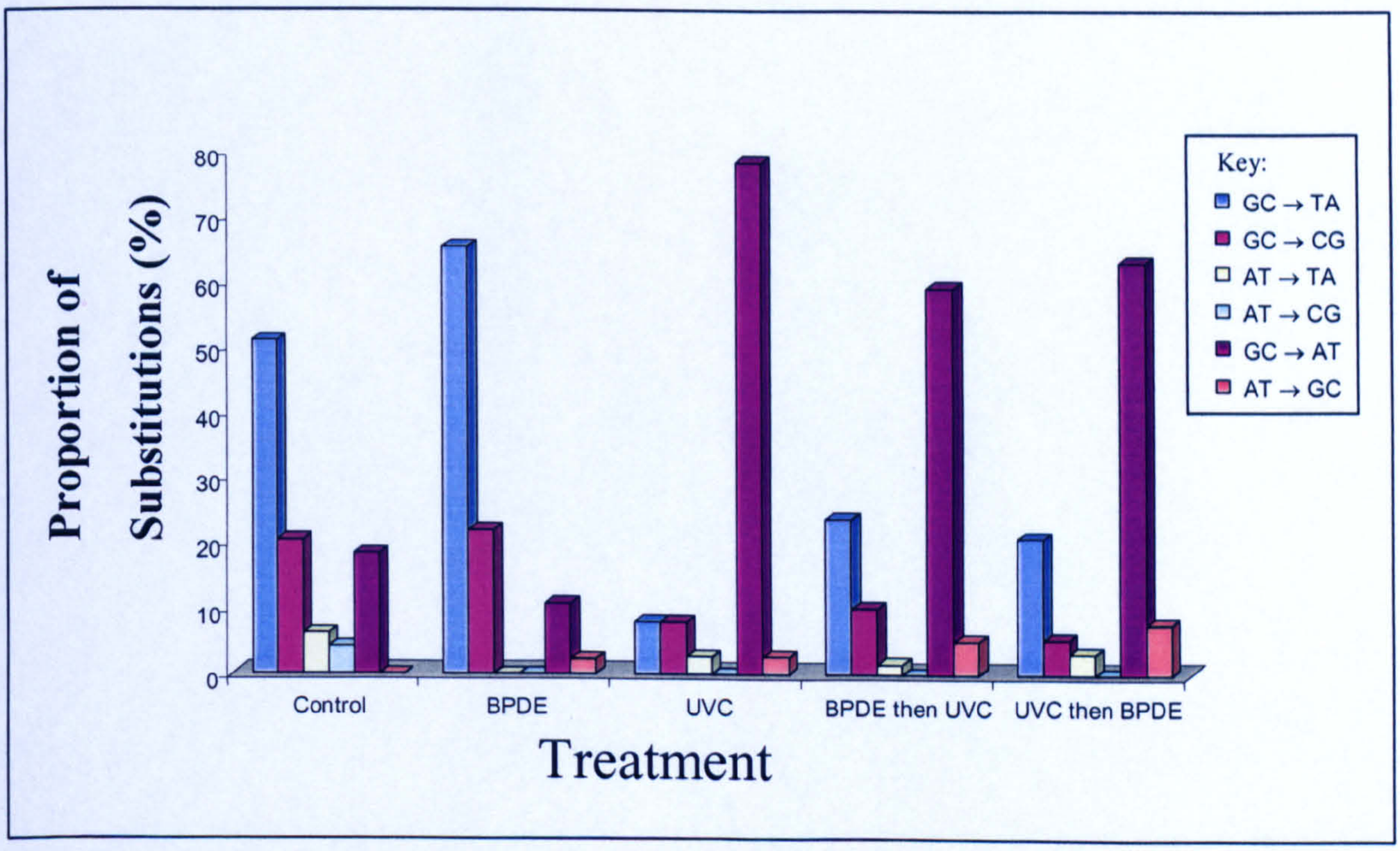


Figure 3.10 Bar chart illustrating the proportion of the different single base or tandem substitution mutations seen in control mutants and in those induced by the various treatments shown. Control mutations taken from Bigger, *et al.*, (1990 and 1992), Boldt, *et al.*, (1991) and, Jeudes and Wogan (1996). BPDE, UVC and BPDE then UVC data is from Part A.

The mutation profile for the multiple substitution spectra are illustrated graphically in Figure 3.11. The relative amounts of the different substitution types differ when plasmid is treated with BPDE first compared to UVC first. The major mutation is the GC→AT transition in each case, but the preference for this mutation, over the GC→TA transversion, is reduced in the UVC first profile compared to the BPDE first profile (greater than 2-fold difference versus almost equal, respectively). The other transversions and transitions, from multiple spectra, also differ in magnitude between the treatments. The relative amounts of multiple substitutions found in the control mutants also differed compared to the single and tandem substitutions.

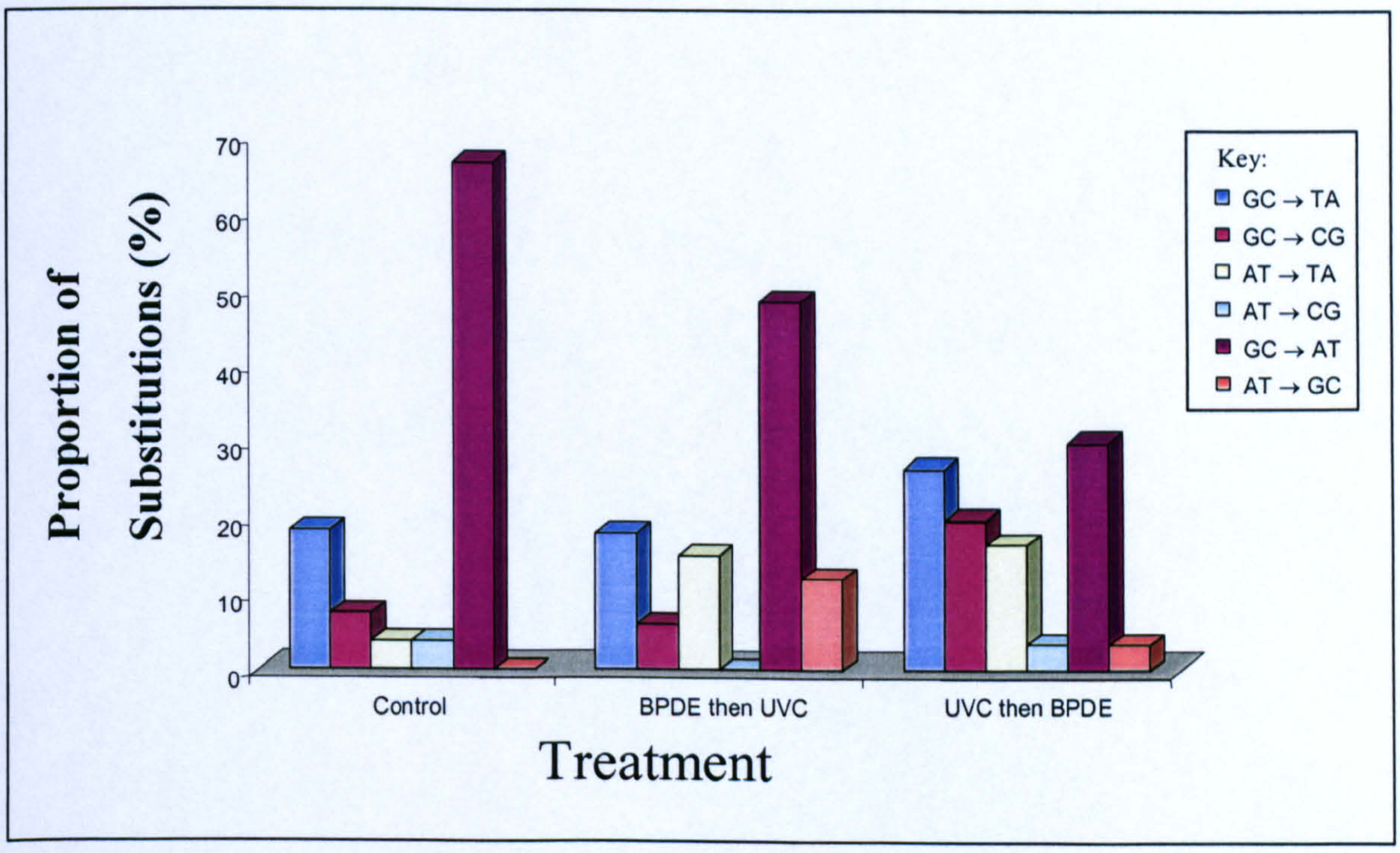


Figure 3.11 Bar chart illustrating the proportion of the different multiple substitution mutations seen in control mutants and in those induced by treatment with BPDE followed by UVC or UVC followed by BPDE. Control mutations taken from Bigger, *et al.,* (1990 and 1992), Boldt, *et al.,* (1991) and, Jeudes and Wogan (1996). BPDE then UVC data is from Part A.

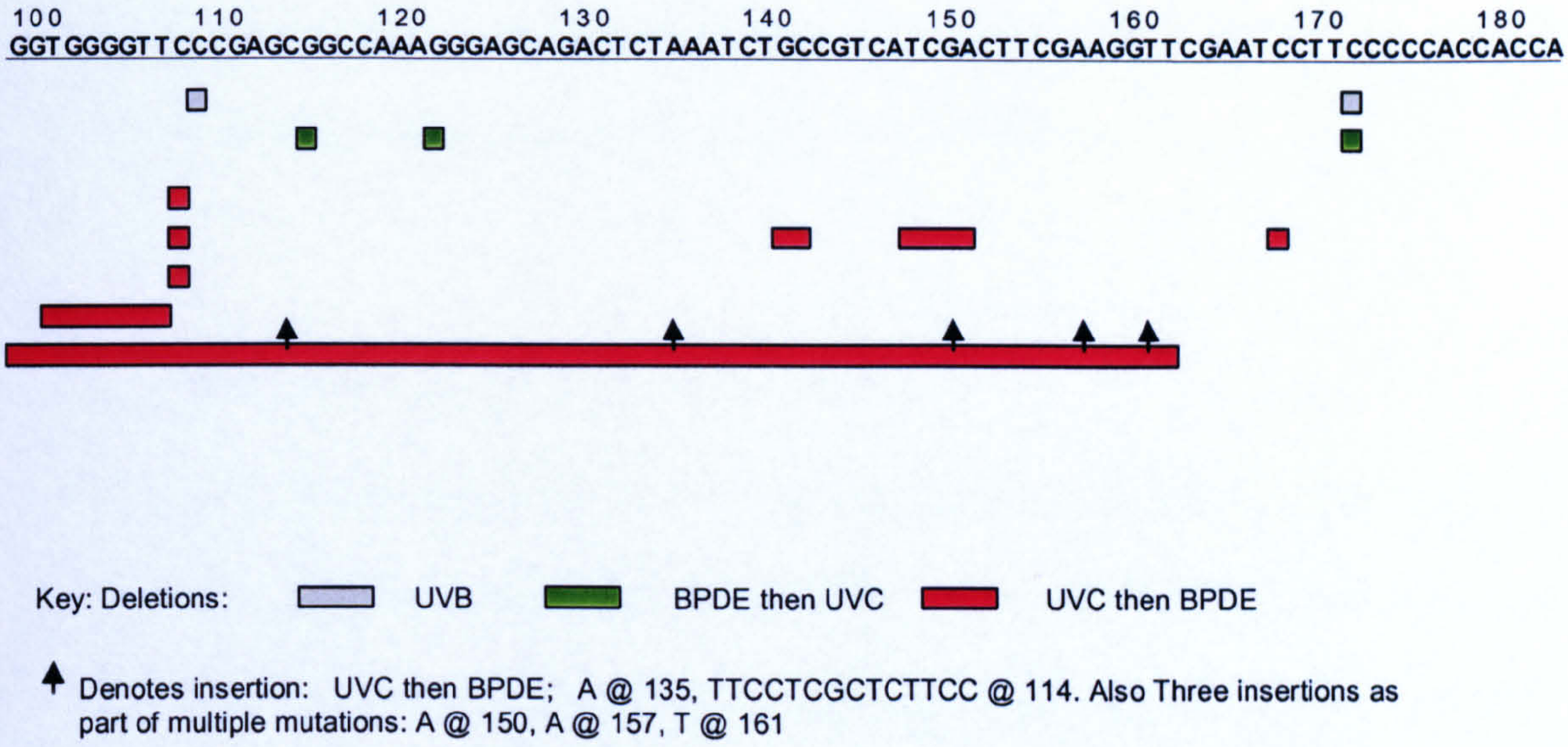


Figure 3.12 Deletions and Insertions induced by UVB, BPDE followed by UVC and UVC followed by BPDE. UVB and BPDE then UVC data is from Part A.

In these experiments (both Part A and B) there were very few frame shift mutations. It was only UVB treatment alone (Part A), BPDE treatment followed by UVC (Part A), and UVC treatment followed by BPDE (Part B) that induced frame shift mutations. Of these, it was only the treatment with UVC before BPDE that induced insertions. Figure 3.12 illustrates these deletions and insertions in the *supF* gene. Treatment with UVB induced two single base deletions; a C in the region 108-110 and a C in the region 172-176. Treatment with BPDE followed by UVC induced three deletions, all of single bases (G at 115/116, G at 122-124, and C in the region 172-176). UVC treatment followed by BPDE induced both single and multiple base deletions. There were three plasmids containing a deletion of C in the region 108-110, and single plasmids containing, TGGGGTT between 101 and 107, GC at 141-142, TCGA between 148 and 151, and C at 168, respectively along with one plasmid which had a long deletion between positions 100 and 162. UVC treatment followed by BPDE induced insertions in two plasmids (an A at position 135, and

TTCCTCGCTCTTCC at position 114) along with three insertions which were part of more complex multiple mutations (A at 150, A at 157, and T at 161).

3.3.2.4 Mutation spectra in *supF* gene

The distribution of single base and tandem substitution mutations within the *supF* gene for plasmids dosed with the various BPDE, UV irradiation or control treatments is shown in Figure 3.13. Treated spectra are found to be significantly different from control (p (same) ≤ 0.05). Mutations are not distributed randomly, but concentrated at one or more sites, known as hotspots.

The hotspot pattern for control, BPDE and UVC treated spectra was described in Section 3.3.1.4. In the mutation spectrum induced by treatment with solvent only there are four hotspots at positions 123, 124, 129, and 163, all of which are at GC basepairs. Treatment of plasmid with BPDE induces hotspots at positions 110, 115 and 175 of the *supF* gene, all of which are also GC basepairs. Treatment of plasmid with UVC also induces hotspots at GC basepairs at positions 104, 123, 156, 159, and 172. When plasmid is treated with BPDE followed by UVC the distribution of single base and tandem substitutions is different to when plasmid is treated with UVC followed by BPDE. There are hotspots at positions 110, 155, 163, 172, and 175 for BPDE treatment followed by UVC irradiation and hotspots at positions 124, 156 and 172 for UVC treatment followed by BPDE. All of these hotspots are at GC basepairs.

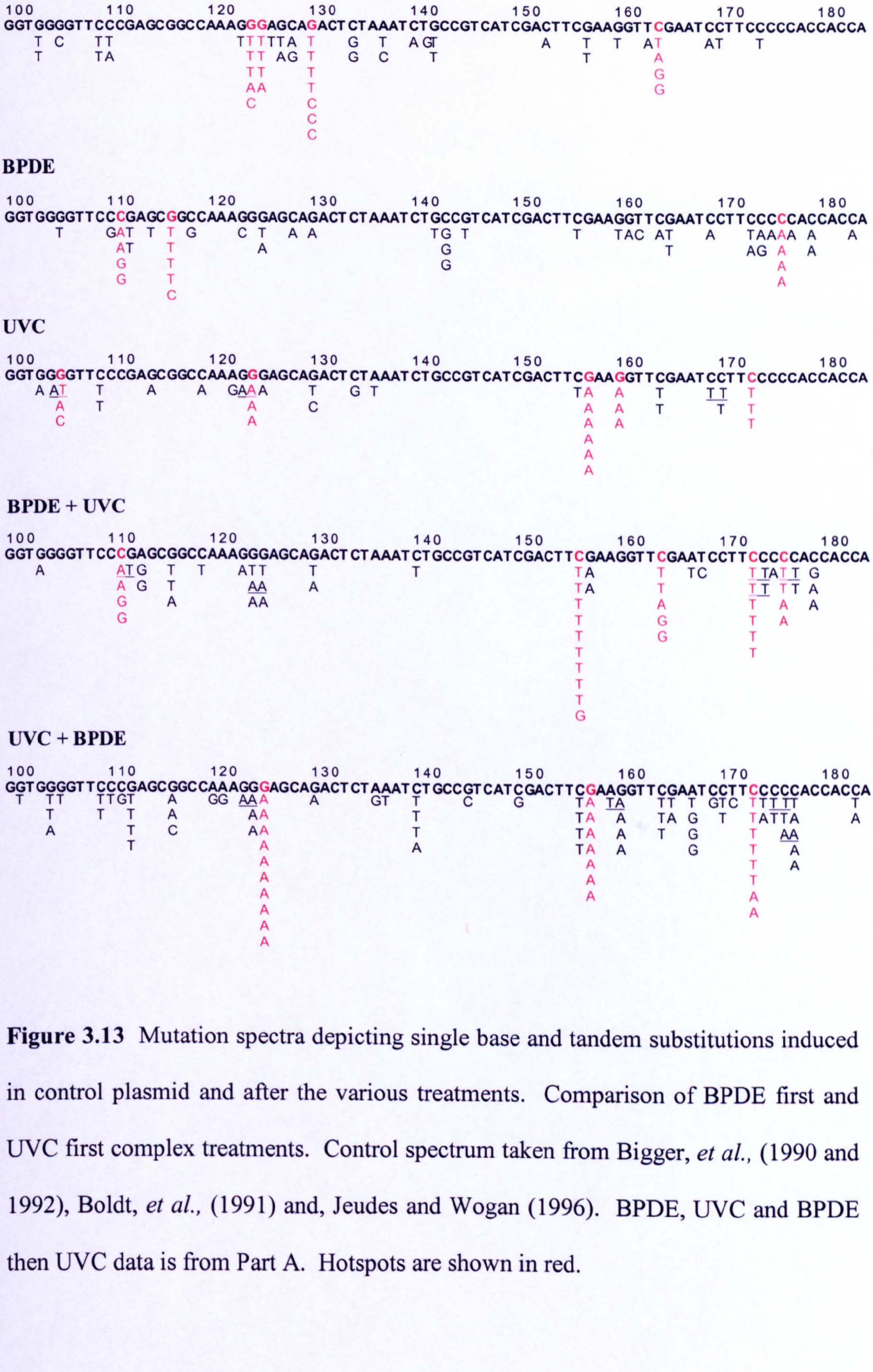


Figure 3.13 Mutation spectra depicting single base and tandem substitutions induced in control plasmid and after the various treatments. Comparison of BPDE first and UVC first complex treatments. Control spectrum taken from Bigger, *et al.,* (1990 and 1992), Boldt, *et al.,* (1991) and, Jeudes and Wogan (1996). BPDE, UVC and BPDE then UVC data is from Part A. Hotspots are shown in red.

The distribution of multiple base substitution mutations within the *supF* gene for plasmids dosed with the BPDE before and after UVC irradiation or control treatments is shown in Figure 3.14. In the control multiple spectrum there are three hotspots at positions 113, 129 and 150, all of which are at GC basepairs. In the spectrum induced by BPDE followed by UVC there are two hotspots at positions 156 and 172. In the spectrum induced by UVC followed by BPDE there is only one hotspot at position 139. All hotspot sites in both single and multiple base substitution mutation spectra were at sites of GC basepairs, for this experiment.

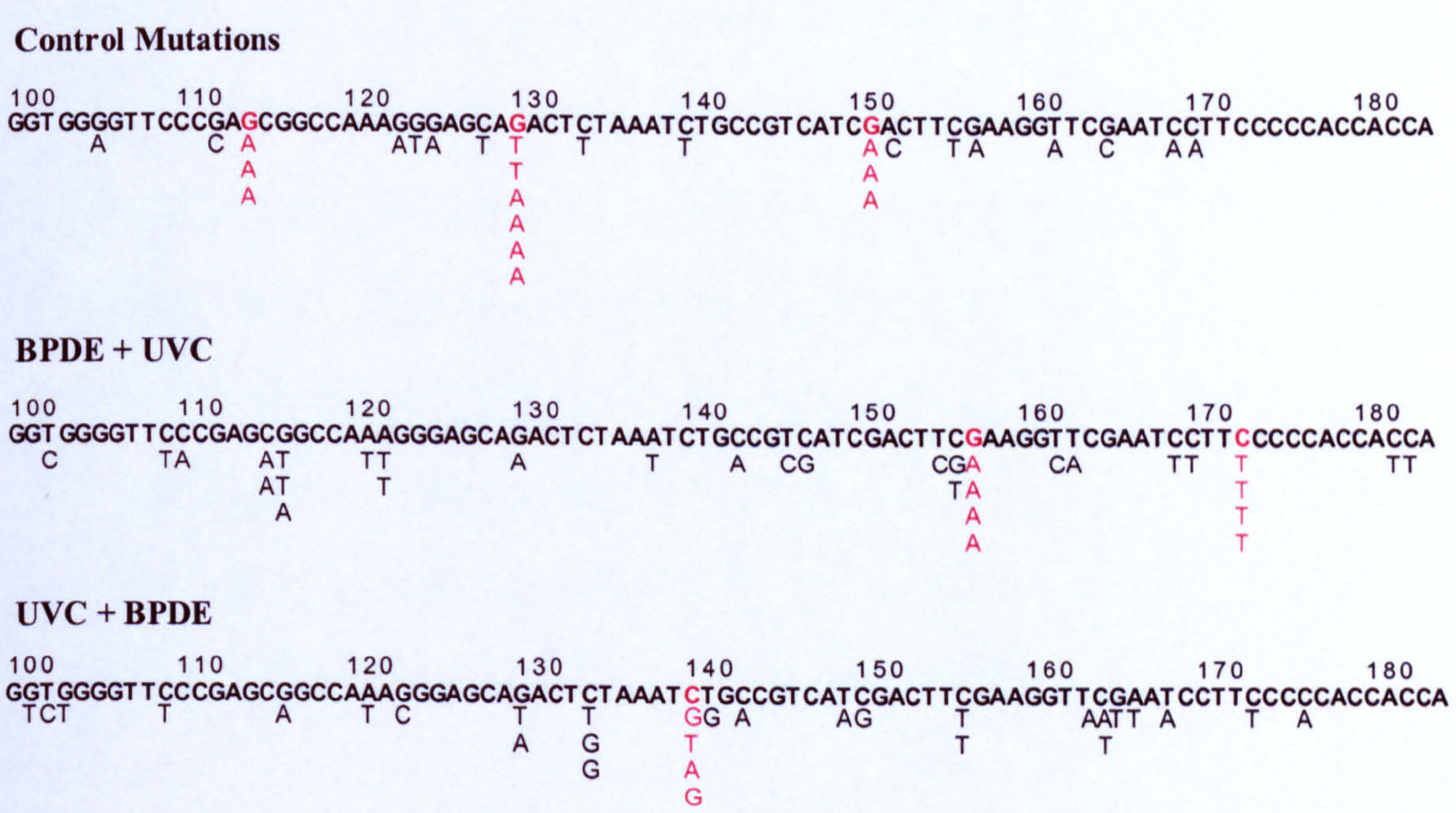


Figure 3.14 Mutation spectra depicting multiple substitutions induced in control plasmids and the comparison of BPDE first and UVC first complex treatments. Control spectrum taken from Bigger, *et al.,* (1990 and 1992), Boldt, *et al.,* (1991) and, Jeudes and Wogan (1996). BPDE then UVC data is from Part A. Hotspots are shown in red.

3.4 Discussion

This chapter presents a comparison of the patterns of mutagenesis induced in the *supF* gene by treatment with UVC (254 nm) radiation, UVB (302 nm) radiation and benzo[*a*]pyrene diol epoxide. Since both BPDE (and other PAH) and UV mutagenesis have been widely studied in the context of the *supF* assay, the primary aim of this study was to evaluate the effects of binary mixtures of mutagens in the *supF* assay. pSP189 plasmid DNA was treated with BPDE, UVB radiation, UVC radiation and combinations of both (BPDE followed by UVB or UVC, and UVB or UVC followed by BPDE) to induce DNA damage. The doses used were 2.4 kJ/m^2 for UVB, 0.63 kJ/m^2 for UVC and 10 μM BPDE along with a control treatment (without UV irradiation or BPDE treatment). In this study the mutation spectra induced when treated plasmid was replicated in human adenovirus transformed kidney (Ad293) cells followed by screening in MBM7070 *E.coli* was examined. Any mutations in the *supF* gene will result in white colonies whilst wild-type colonies will be blue. Mutant colonies were grown and sequenced to yield a distribution of mutations induced by the various treatments.

There were two reasons for choosing the combination of BPDE and UV exposure. Firstly, the types of mutations induced by BPDE and UV irradiation are quite distinct. The most common mutations induced by BPDE in a number of mutation assays are GC→TA transversions (Maher, *et al.*, 1989; Kohler, *et al.*, 1991), whilst GC→AT mutations are the most common base substitutions induced by UV irradiation (Miller, 1985; Bredberg, et al., 1986). These mutations arise as a result of damage to purines, chiefly guanine, in the case of BPDE (Weinstein, et al., 1976; Maher, *et al.*, 1989),

and pyrimidines in the case of UV (Protic-Sabljic, *et al.*, 1986; Otoshi, *et al.*, 2000). Hence it would be possible to distinguish between the mutagenic contributions of the two agents in a dual exposure protocol. Secondly, there is evidence in aquatic organisms that the toxicity of PAH pollutants is enhanced by UV irradiation (Nikkila, *et al.*, 1999; Boese, *et al.*, 2000; Laycock, *et al.*, 2000). There is, therefore, some rationale to thinking that DNA damaged by a PAH such as BPDE may be more susceptible to UV induced damage or mutations.

3.4.1 Part A: Treatment with BPDE followed by UVB or UVC radiation

Plasmid was treated with BPDE followed by UV irradiation and this showed that the mutation frequency increased dramatically in the plasmid treated with combined exposures. Treatment of plasmid with BPDE and UVB caused an increase in mutation frequency almost 4000-fold greater than control, approximately 100-fold greater than BPDE treatment alone and 2.6-times higher than with UVB irradiation alone. This increase was more than an additive effect; it revealed a synergistic relationship between the treatments. There are a number of possible explanations for the increased mutation frequency observed when adducted DNA is UV irradiated: (i) GC→TA mutations are induced by oxidative DNA damage, and the increased mutation frequency is a consequence of reactive oxygen species produced from the interaction between the BPDE adduct and UV radiation, (ii) the presence of BPDE adducts could cause the DNA to absorb more energy from the UV radiation, resulting in enhanced UV damage, which may potentially be targeted around the sites of BPDE

adduct formation, (iii) UV irradiation of the BPDE adduct may generate a photoactivation product that is inherently more mutagenic than the original adduct, (iv) the presence of UV and BPDE adducts in the same DNA molecule alters the mutagenicity of one or the other.

Mutations induced by separate treatments with BPDE and UV irradiation have previously been reported in the *supF* system (Yang, et al., 1987; Maher, *et al.*, 1989; Bredberg, et al., 1986; Parris and Seidman, 1992). In this case the BPDE induced mutation frequency was about five-fold higher for a similar adduct level quantified by ^{32}P-postlabelling, compared to that reported in two previous studies (Yang, *et al.*, 1987; Maher, *et al.*, 1989). However, the spectrum of mutation types was similar in all three studies with GC→TA, GC→AT and GC→CG mutations accounting for 63 %, 8 % and 18 %, respectively in Yang *et al.*, 63 %, 9 % and 18 %, in Maher, *et al.* and for 65 %, 11 % and 22 %, in this study. The distribution of mutations, however, was quite different, with no common mutation hotspots apparent in the spectra. The sites of mutation hotspots in this study, 110, 115 and 175 were not hotspots in the two published studies. However, these hotspot sites in the current study all correlate with hotspots for BPDE adduction in the *supF* gene, as determined by a polymerase stop assay in the study by Maher, *et al.*, (1989). Interestingly, this group reported that hotspots of damage and hotspots of mutation did not correlate. Use of the polymerase stop assay has also shown that positions 110 and 175 are potential hotspot sites for BPDE damage (Ross, *et al.*, 1994). The polymerase stop assay was not performed as part of this project, so no definite conclusions can be made regarding the distribution of adduct and mutation hotspots. Differences in the distribution of mutations between this study and the earlier studies are possibly due to the fact that some of the plasmid

sequence in pSP189 differs from that of pZ189 and pS189 used in the earlier studies. Differences in induced mutation spectra in pS189 (an earlier version of pSP189) and pZ189 have been reported for aflatoxin B_1 (Courtemanche and Anderson, 1994) and it is known that alterations in DNA sequence some distance from the target gene can modulate mutational spectra (Rodriguez and Loechler, 1993a; Levy, *et al.*, 1996a&b).

The sites of mutation hotspots in the mutation spectrum induced by UVC (254 nm) radiation (positions 104, 123, 156 and 159) closely match those seen in African green monkey kidney cells (Keyse, *et al.*, 1988). In addition, though, there are a number of extra mutation hotspots, at positions 108, 109, 111, 113, 168 and 169 in the monkey cells which are not observed in Ad293 cells and a hotspot in Ad293 cells not seen in monkey cells (at position 172). In human excision repair deficient (XP12BE, from *Xeroderma pigmentosum* patient) fibroblast cells, mutation hotspots are present at positions 123, 156, 168 and 169 (Bredberg, *et al.*, 1986) whereas, using repair proficient fibroblasts hotspots were seen at positions 123 and 156 only. In another study, carried out in human XP12BE cells, UVC induced hotspots were seen at positions 99, 103, 108, 122, 124, 155, 156, 164, and 168 (Parris and Seidman, 1992). A mutation spectrum has also previously been published for UVB (313 nm) radiation (Keyse, *et al.*, 1988) which demonstrates hotspots at positions 108, 109, 111, 118, 122, 133, 139, 149, 155, 156, 159, 164, and 168, some of which match the hotspots found in the present study at positions 108, 122, 155, 156, 159 but not at the position 172. This hotspot, at position 172, was seen for both UVC and UVB irradiation of pSP189 plasmid. There are some similarities between the studies, especially the fact that hotpots are almost always at GC basepairs. Differences in the positions of hotspots may be due to the differential repair abilities (repair deficient versus repair

proficient), cell type (fibroblasts versus kidney cells) and species (monkey versus human) of the cell lines used in this study in comparison to those used previously. The differences in mutation distribution may also be due to the fact that slightly different plasmids were used; pZ189 in studies by Bredberg, *et al* (1986) and Keyse *et al* (1988) and pSP189 in the study by Parris and Seidman (1992) and in the present study.

The distribution of mutations seen for individual BPDE treatment and UV irradiation were noticeably different to each other. However, it seems that when BPDE and UVC treatments were combined the resulting mutation spectra were also combined. Hotspots observed with the single agent exposure were also seen in the mutation spectrum from the combined BPDE and UVC treatments (positions 110 and 175 from BPDE and 172 from UVC), which also contained a new hotspot only seen with exposure to both mutagens (163). This was not the case for the combined treatments of BPDE and UVB, where there is little evidence of BPDE induced mutation hotpots, since three out of the four hotspots were also present in the UVB mutation spectrum. The fourth hotspot, at position 163, was also seen in the combined treatment of BPDE followed by UVC, which suggests that this may have been caused as a result of increased mutagenicity of any photoactivated BPDE lesions created.

Single and multiple base substitutions have been presented on separate spectra because it has been suggested that multiple mutations arise through a different mechanism to single base mutations (Courtemanche and Anderson, 1999). It has previously been reported that multiple mutations correlate with increased levels of single strand breaks in plasmid DNA, either as a consequence of treatment or during

repair of induced lesions in repair competent cells (Seidman, *et al.*, 1987). An increased frequency of DNA strand breaks has been demonstrated in a strand break assay, when BPDE treated pUC18 plasmid was irradiated with both UVB and UVC compared to UVB or UVC treatment alone (Routledge, *et al.*, 2001). Multiple mutations were seen preferentially after treatment with combined doses of BPDE and UV in this study, so this may be due to an increased amount of strand breaks in the combined treatment. However, since the strand break assay was not repeated using the pSP189 plasmid, the absolute relationship between strand breaks and BPDE/UV in this system remains to be established. There was a very low amount of frameshift mutations seen for all treatments of plasmid in this study (only five deletions seen after treatment, 2 in UVB and 3 in BPDE + UVC). Frameshift mutations, would also, presumably imply the presence of strand breaks in the plasmid (Kanbashi, *et al.*, 1997), where the nicked strand is ligated incorrectly during repair. The lack of frameshift mutations may, therefore, suggest that strand breaks are easily repaired in this context and so regarded as unimportant by the cells used.

Comparison of the mutation spectra generated in this study, indicates that in the BPDE and UV spectra, the BPDE adducts have contributed to the increased frequency of mutations in the combination treatment of BPDE and UV versus BPDE alone, even though the level of BPDE adducts has not altered, as the same BPDE treated plasmid sample was used for both combined treatments. This evidence suggests that the BPDE adducts have themselves become more mutagenic, possibly due to photoactivation of the adduct, or to effects of UV adducts nearby (i.e. a change in DNA conformation caused by the presence of a UV adduct has led to a BPDE adduct becoming more mutagenic than before). The issue of what factors may influence the

induction of different mutations by the same adduct forming agent, or even by the same specific adduct, under different circumstances, has been examined by Loechler and colleagues (Rodriguez, and Loechler, 1993a&b; Seo, *et al.*, 2000), using BPDE adducts as an example. They have provided evidence that the same major adduct of (+)-anti-BPDE, formed by *trans* addition of N^2-dG to C10 of (+)-anti-BPDE, can be locked into two conformations, one that leads to G→T mutation, and another that leads to G→A mutation. It is therefore possible that UV irradiation of BPDE adducted DNA causes conformational switching of specific adducts that leads to changes in mutation spectra.

Whilst these results may relate specifically to the interaction of UV irradiation with BPDE adducted DNA, the work described in this chapter was inspired by an interest in the potential mutagenic effects of multiple exposures that may result from the presence of more than one type DNA lesion. Another potential example of interaction between two types of DNA damage which relates to the tamoxifen work reported in this thesis (Chapter 4) comes from an earlier study of the mutagenicity of the tamoxifen derivatives, α-acetoxytamoxifen and 4-hydroxytamoxifen (Lowes, *et al.*, 1999). In order to activate 4-hydroxytamoxifen to the DNA active 4-hydroxytamoxifen quinone methide, 4-hydroxytamoxifen was incubated with horseradish peroxidase (HRP) and hydrogen peroxide. As this activation step itself generates mutagenic oxidative DNA lesions, the HRP/ H_2O_2 treatment without 4-hydroxytamoxifen was used as a control in a *lacI* gene bacterial mutagenicity assay. Activated 4-hydroxytamoxifen induced mutations at a frequency approximately sixteen-fold higher than untreated control, and the HRP/ H_2O_2 treatment itself induced mutations at a frequency about seven-fold higher than control. It was also

demonstrated that the mutagenicity of the HRP/ H_2O_2 activated 4-hydroxytamoxifen was two orders of magnitude higher than that of α-acetoxytamoxifen in the same system. The authors concluded that the adducts formed by 4-hydroxytamoxifen quinone methide are more mutagenic than the adducts formed by α-acetoxytamoxifen. However, considering the BPDE/UV results presented here, it may also be possible the higher mutagenicity of HRP/ H_2O_2 activated 4-hydroxytamoxifen can be attributed to some extent to the oxidative lesions enhancing the mutagenicity of the tamoxifen adducts, or *vice versa*. This could be investigated by reacting plasmid with α-hydroxy-4-hydroxytamoxifen, a tamoxifen metabolite which forms the same major adducts as 4-hydroxytamoxifen quinone methide, but doesn't require activation by HRP/ H_2O_2. Analysis of the mutations induced by this compound in the *supF* assay may therefore indicate whether the adducts are inherently more mutagenic than the α-acetoxytamoxifen adducts or if the combined exposure is having a significant effect.

If the presence of UV lesions and BPDE adducts on DNA does influence the mutagenicity of one or more of these adducts, it follows that mutations induced by agents that cause a range of adducts on DNA might demonstrate a similar effect. This would have implications for the conclusions drawn from experiments in which the mutagenicity of single DNA adducts are assessed in site-specific assays and indicates the need for more complex systems where the effects of multiple site-specific adducts are investigated.

3.4.2 Part B: Treatment with UVB or UVC radiation followed by BPDE

To begin to address the questions posed by the results seen after BPDE followed by UV exposure was investigated, the experiments were repeated with plasmid that had been irradiated with UV first, then treated with BPDE. Combined treatment of BPDE and UV radiation resulted in a mutation frequency increase which was greater than the sum of the mutation frequencies from separate treatments. If this increase was due to the proximity of BPDE adducts and UV photoproducts then the mutation frequency should be the same when the treatment is reversed, i.e. UV treatment followed by BPDE treatment. A difference in mutation frequency for this experiment would suggest that the BPDE adducts are being activated by the UV irradiation.

It is interesting to note that when the level of DNA damage was quantified, using ^{32}P-postlabelling, it was found that during the combined treatments (both BPDE followed by UV and vice versa) there was an apparent increased amount of DNA lesions which could be labelled. This extra number of lesions may be due to the production of photoactivated BPDE adducts which are more efficiently labelled, which may be due to differences in structure. The concentration of BPDE used for each plasmid sample was constant and reactions were carried out at the same time, so any experimental error should have been minimized. The mutation frequency does vary between the two experiments, especially for UVB treatments, but there is an increase in both sets of data when UV and BPDE treatments are combined. This evidence of chemical change in the DNA lesions being detected, along with the fact that combined treatments are more mutagenic than single treatments, merits further experimentation. Although, there was a difference in the number of DNA adducts, as quantified by ^{32}P-

postlabelling, between the two BPDE treatments in Parts A (2.2 per 10^4 nucleotides) and B (0.5 per 10^5 nucleotides), the mutation frequency induced in the *supF* assay by both these treated plasmid samples was similar (12×10^{-4} in Part A and 14×10^{-4} in Part B). Since a standard was not available to correct for experimental variation, the difference in adduct levels is probably due to variables in the ^{32}P-postlabelling assay (e.g. enzyme efficiencies).

A mutation spectrum was compiled for the treatment with UVC followed by BPDE, so that comparisons of the types and distribution of mutations can be made with that seen for the treatment with BPDE followed by UVC. The results revealed that the types of mutations did not really differ. Single base substitutions were the major type of mutations seen for both treatments, with these being mainly transitions at GC basepairs (59% GC→AT for BPDE followed by UVC, and 63% GC→AT for UVC followed by BPDE). However, there is a noticeable difference in the types of multiple mutations observed, as multiple mutations are twice as frequent in treatment with BPDE followed by UVC than UVC followed by BPDE. Treatment with BPDE followed by UVC induces mainly transitions (48% as GC→AT, 12% as AT→GC, 61% overall), which are typical of UV mutagenesis. UVC treatment followed by BPDE treatment, on the other hand, predominantly induces transversions, the typical mutations induced by bulky PAH's; 67% of all multiple mutations were transversions, 27% as GC→TA, 20% as GC→CG, 17% as AT→TA and 3% as AT→CG. The addition of a pretreatment may somehow sensitize the plasmid to the damage which is induced by the second treatment.

Compared to when plasmid is treated with BPDE followed by UV irradiation, UV irradiation does not induce such a marked increase in mutation frequency over the single treatments and control. The increases in mutation frequencies are still greater than additive, but in this experiment the maximum mutation frequency increase was in the order of 59-fold (UVC then BPDE) rather than almost 657-fold (BPDE then UVC) as seen before (Part A). Although slight variations in mutation frequency will be due to the fact that these experiments were carried out on different occasions, the magnitude of difference in mutation frequency, 118-fold increase for BPDE followed by UVB (Part A) than for UVB followed by BPDE (Part B), and 11-fold increase for BPDE followed by UVC (Part A) than for UVC followed by BPDE (Part B), suggests that some sort of chemical reaction is taking place. The UV irradiation of the BPDE DNA adducts has resulted in production of new DNA lesions which are more mutagenic than both those induced by UV irradiation or BPDE treatment alone. Of course, there is still the possibility of reactive oxygen species being produced by the combination of UV and BPDE together. Judging by the mutation frequencies it is not just the fact that UV and BPDE adducts are formed together on the same plasmid which increases the mutation frequency, but some more complex synergistic effect. Further work investigating the structural changes BPDE adducts undergo when irradiated with UV may provide some insight into the mechanisms responsible for these effects. The realization that UV irradiation of PAH increases both their mutagenicities could be important in the use of topical eczema, psoriasis or other skin disorder treatments, regulation of environmental pollutants, such as in aquatic organisms, or any situations where there is exposure to mixtures of carcinogens.

Chapter 4

Mutations induced in the pSP189 *supF* gene by metabolites of tamoxifen

4.1 Introduction

4.1.1 Breast cancer

Breast cancer is by far the most common form of cancer in women in the UK, accounting for 30% of all new cases of cancer in women (Cancer Research UK, www.cancerresearchuk.org). Approximately 1 in 9 women will develop breast cancer at some stage during their lives, resulting in the deaths of nearly 13,000 women each year. The incidence of breast cancer increases with age (Pike, *et al.*, 1993); females below 30 years of age are rarely diagnosed with breast cancer (218 new cases in 1997) whilst women most at risk are in the 50-54 age bracket (over 5000 diagnoses will be made per year {5010 new cases in 1997}) (www.cancerresearchuk.org).

There are many risk factors for breast cancer; these include family history and inheritance of mutations in tumour suppressor genes, which confer an increased risk of cancer, such as BRCA1 and BRCA2 (Blackwood and Weber, 1998). Exposure to oestrogen for a longer than average time period also confers increased risk, such as in cases of early menarche, late first pregnancy, and late menopause. Over eating and lack of exercise in children can result in increased growth which can then also result in early menarche thus contributing to breast cancer risk. Diet can also be a risk factor, especially fat intake and alcohol (www.cancerresearchuk.org).

Although the incidence of breast cancer continues to increase yearly in the UK, deaths from breast cancer in the UK and USA have been decreasing substantially since 1987 (Peto, *et al.*, 2000). This decrease can be attributed to improvements in the way in which breast cancer is diagnosed and treated. This includes the use of mammography

screening and the introduction, and then increased use of hormonal and cytotoxic treatments for breast cancer, such as tamoxifen.

4.1.2 Tamoxifen

Tamoxifen [*trans*- (*Z*)-1-[4-[2-(dimethylamino) ethoxy] phenyl]-1,2-diphenyl-1-butene] is a non-steroidal anti-oestrogenic drug which has been used in the adjuvant therapy of breast cancer for almost 30 years. More recently it has been approved by the United States Food and Drug Administration (FDA) for use as a chemopreventative agent in women at high risk of breast cancer. This decision was based on the observation that tamoxifen decreases contralateral breast cancer in treated women by 39% (Early Breast Cancer Trialist's Collaborative Group, 1992) and by the finding that tamoxifen achieved a statistically significant 49% reduction in the incidence of invasive breast cancer in healthy women with increased risk of the disease (Fisher, *et al.*, 1998).

However, both chemopreventative and therapeutic dosing strategies have been shown to cause a small but significant increase in the risk of endometrial cancer in comparison to untreated women (Fornander, *et al.*, 1989; Fisher, *et al.*, 1994). In addition, both short-term and long-term treatment of rats with tamoxifen results in the induction of hepatocellular carcinomas preceded by the formation of high levels of hepatic DNA adducts (Han and Liehr, 1992; Greaves, *et al.*, 1993; Carthew, *et al.*, 1995a). Tamoxifen DNA adducts have also been reported in leucocytes and endometrial tissue of women undergoing tamoxifen therapy (Hemminki, *et al.*, 1997; Shibutani, *et al.*, 1999), adding to concerns over the long-term health hazards of tamoxifen.

4.1.3 Activation and detoxication of tamoxifen

In rats, tamoxifen is believed to induce cancer through a genotoxic mechanism involving the formation of DNA adducts. Tamoxifen is not itself reactive, like many carcinogens it requires metabolism to electrophilic intermediates before binding to DNA can occur. The metabolism of tamoxifen (Figure 4.1) has been shown to be qualitatively similar in humans, mice and rats, using *in vitro* liver microsomal systems (Ruenitz, *et al.*, 1984; Lim, *et al.*, 1994; Jones, *et al.*, 1996). Phase I metabolism, by components of the cytochrome P-450 mixed function oxidase system, produces the major metabolites *N*-desmethyltamoxifen, 4-hydroxytamoxifen and tamoxifen *N*-oxide. The most important enzyme for carrying out the *N*-demethylation reaction in human liver is CYP3A4 (Jacolot, *et al.*, 1991; Berthou, *et al.*, 1994). 4-Hydroxylation is carried out by CYP2D6 (Dehal and Kupfer, 1997), although there is evidence that other CYP isoenzymes may also take part (Crewe, *et al.*, 1997). Tamoxifen *N*-oxide is produced by flavin mono oxygenase (FMO) (Mani, *et al.*, 1993). 4-Hydroxytamoxifen and *N*-desmethyltamoxifen are thought to be further metabolised to 3,4-dihydroxytamoxifen and *N,N*-didesmethyltamoxifen, respectively (Dehal and Kupfer, 1996; Poon, *et al.*, 1995).

Figure 4.1. (overleaf) Tamoxifen is metabolised in humans by cytochrome P450 mixed function oxygenases via *N*-demethylation by CYP3A4, 3 / 4-hydroxylation by CYP2D6, α-hydroxylation by CYP3A4 and *N*-oxidation by FMO (flavin mono oxygenase). There is evidence that other CYP isoenzymes may also take part.

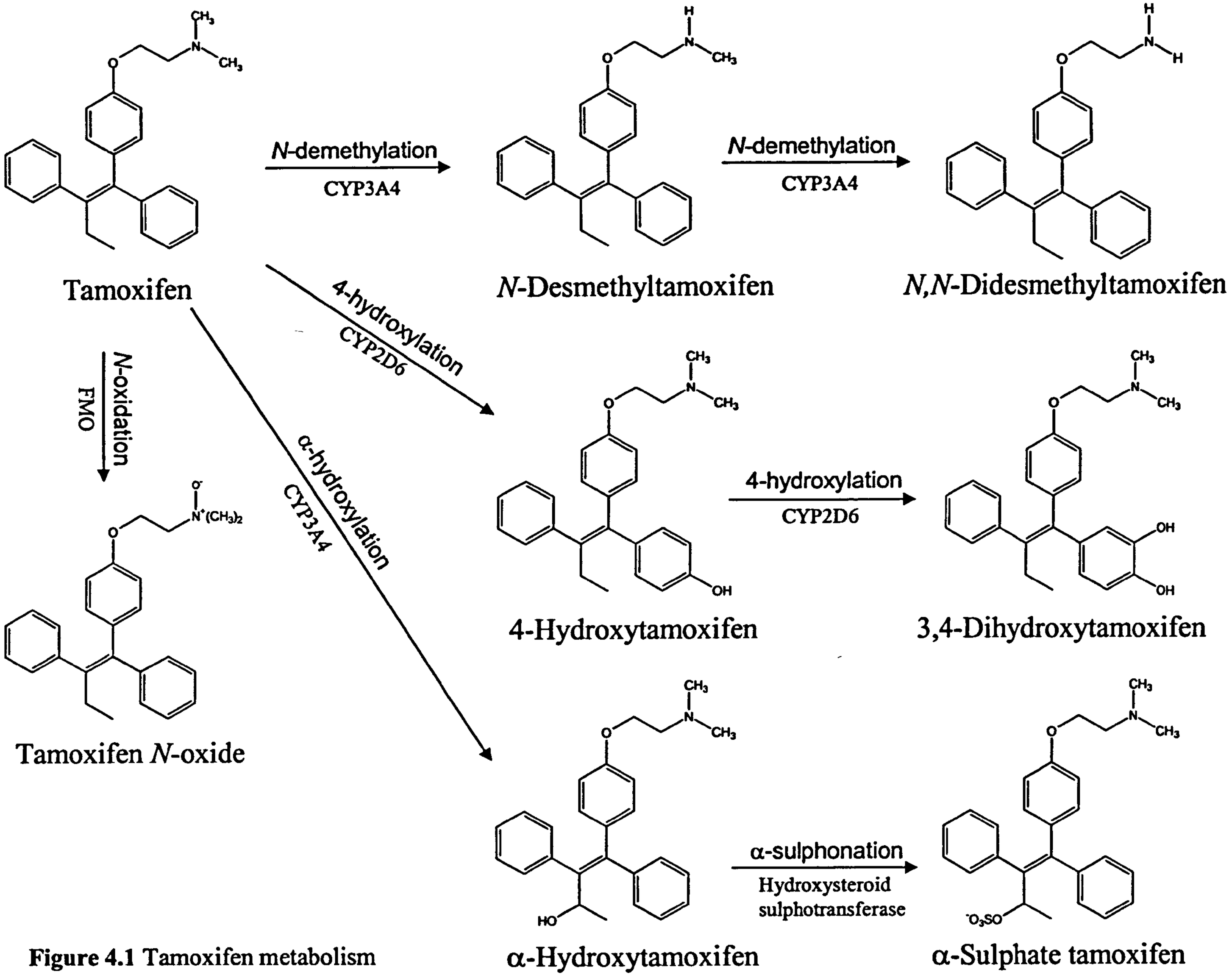
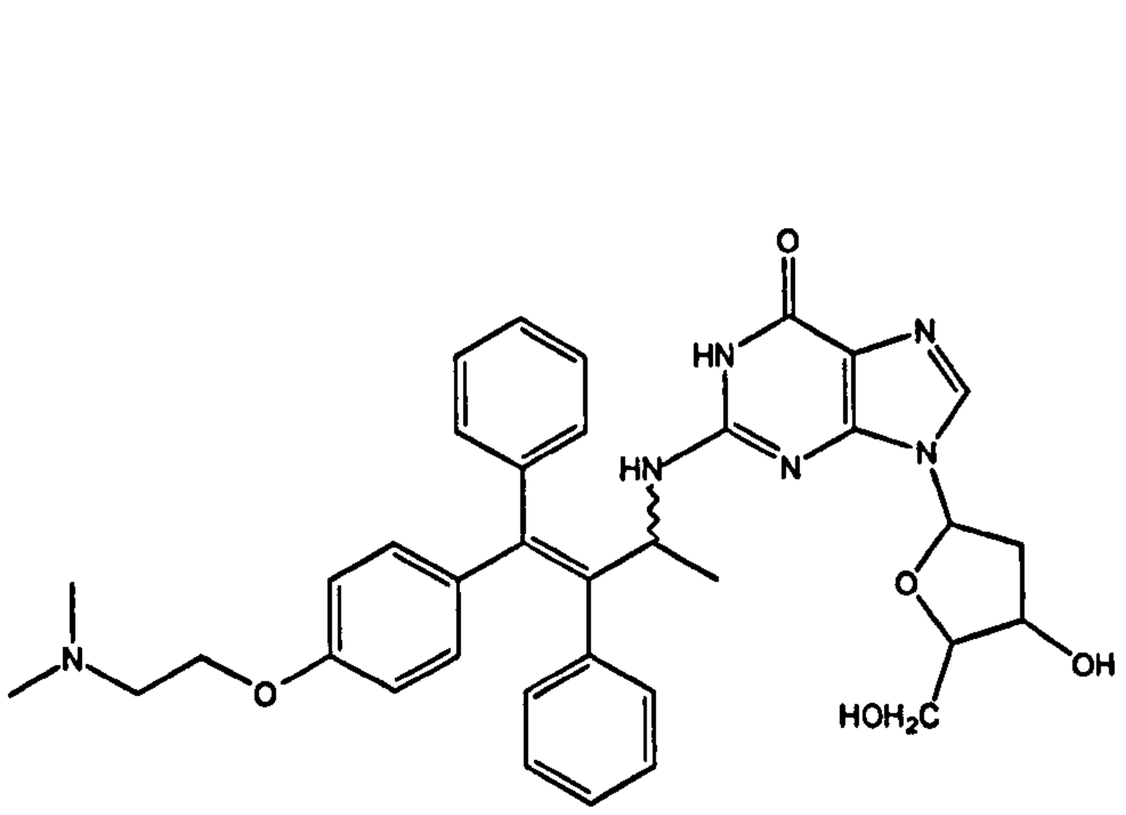


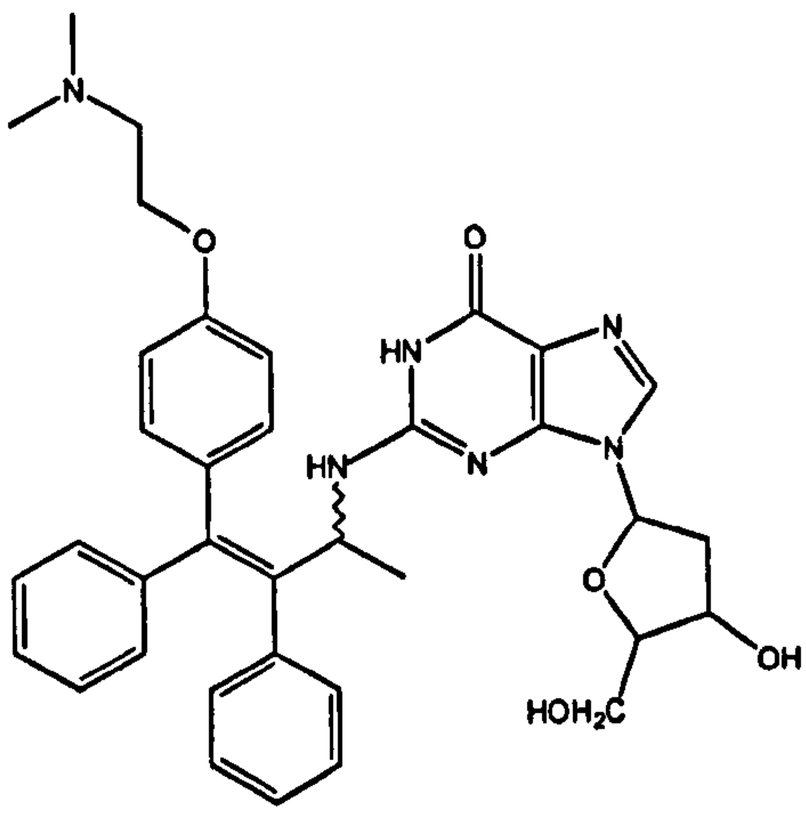
Figure 4.1 Tamoxifen metabolism

Although *N*-desmethyltamoxifen, 4-hydroxytamoxifen and tamoxifen *N*-oxide are the major metabolites *in vivo*, it is one of the minor metabolites, formed by α-hydroxylation (Phillips, *et al.*, 1994; Potter, *et al.*, 1994; Jarman, *et al.*, 1995; Phillips, *et al.*, 1996) which is responsible for the major tamoxifen DNA adduct seen in rats, mice and humans (Osborne, *et al.*, 1996&1997; Dasaradhi and Shibutani, 1997). α-Hydroxytamoxifen has been found in the plasma of women treated with tamoxifen (Poon, *et al.*, 1995) and as the glucuronide in the bile of tamoxifen treated rats, where it accounts for around 0.1% of the administered dose (Jacolot, *et al.*, 1991). α-Hydroxytamoxifen is produced by CYP3A4 (Boocock, *et al.*, 2002). It is activated by the phase II enzyme hydroxysteroid sulphotransferase via the conjugation of a sulphate ion (Shibutani, *et al.*, 1998; Davis, *et al.*, 1998). Further loss of this sulphate moiety leaves a carbocation (Sanchez, *et al.*, 1998), promoting nucleophilic attack by the N^2 amino group of deoxyguanosine to form α-(deoxyguanosin-N^2-yl)-tamoxifen(Osborne, *et al.*, 1996; Rajaniemi, *et al.*, 1999). This adduct exists as four diastereoisomers, with the *trans*-forms accounting for one of the major adducts in rat liver DNA. *N*-Desmethyltamoxifen is similarly thought to undergo α-hydroxylation followed by sulphonation to form a second major adduct in the liver of tamoxifen treated rats, α-(deoxyguanosin-N^2-yl)-*N*-desmethyltamoxifen (Brown, *et al.*, 1999; Phillips, *et al.*, 1999; Rajaniemi, *et al.*, 1999; da Costa, *et al.*, 2000). Another major tamoxifen metabolite, 4-hydroxytamoxifen (Randerath, *et al.*, 1994), is also believed to be activated to a DNA reactive species either via α-hydroxylation, presumably followed by α-sulphonation, or possibly via formation of a quinone methide or quinone species (Marques and Beland, 1997). Studies in rats have reported the formation of DNA adducts derived from 4-hydroxytamoxifen (Pathak, *et al.*, 1995)

although in rat liver these have been detected at much lower levels than those derived from α-hydroxytamoxifen (Martin, *et al.*, 1998).



α-(deoxyguanosin-N^2-yl)-*trans*-tamoxifen



α-(deoxyguanosin-N^2-yl)-*cis*-tamoxifen

Figure 4.2 Tamoxifen can form *cis* or *trans* adducts with DNA. The major tamoxifen-deoxyguanosine adducts is shown, adducts of demethylated or 3 or 4-hydroxylated tamoxifen can also be formed

Metabolism of tamoxifen to α-hydroxytamoxifen occurs at a three-fold higher rate in rats compared to man (Shibutani, *et al.*, 1998; Boocock, *et al.*, 1999) and sulphonation occurs at a rate five-fold higher in rats than man (Boocock, *et al.*, 2000). As well as being activated at a much reduced rate in humans, tamoxifen is also detoxicated, via glucuronidation to form α-hydroxytamoxifen glucuronide, at a far greater rate in humans than in rats (10-100 fold increase) (Boocock, *et al.*, 2000). So although tamoxifen induces hepatocellular carcinomas in rats, there is a safety factor involved for humans, which should explain the fact that, although there have been some case reports of acute liver toxicity caused by tamoxifen (Ching, *et al.*, 1992) there has, to

date, not been any increase in the incidence of liver cancers among tamoxifen treated women (Fornander, *et al.,* 1989; Rutqvist, *et al.,* 1995; Clarke, *et al.,* 1998).

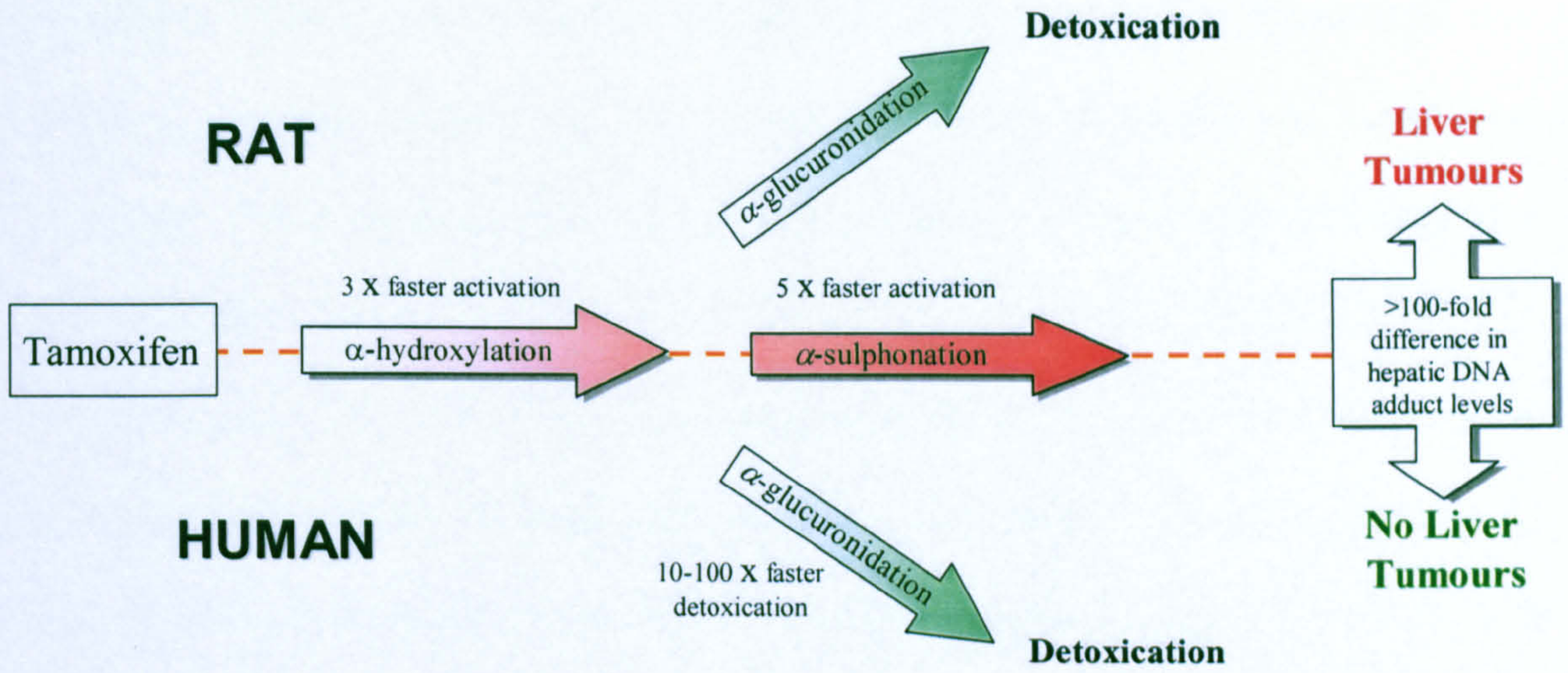


Figure 4.3. Illustration of relative risk in rats and women treated with tamoxifen (Adapted from; Brown, 2002).

4.1.4 Carcinogenicity of tamoxifen in rodents

Tamoxifen is a potent rat liver carcinogen since both short term (Carthew, *et al.,* 1995a) and lifetime (Greaves, *et al.,* 1993) exposure of rats to tamoxifen results in an increase in the incidence of hepatocellular tumours. This does not appear to occur via an oestrogen-receptor (ER)-based mechanism as the incidence of tumours was similar in both male and female rats, showing a dose dependent increase over the range 5-35 mg/kg/day (Greaves, *et al.*, 1993). In comparison, it was found that mice were resistant to tamoxifen (Tucker, *et al.,* 1984; Martin, *et al.,* 1997) as tumours are not formed. Tamoxifen does not act as a tumour initiator if given as a single dose to rats (Carthew, *et al.,* 1995a) but does promote hepatocellular tumours when initiated by *N*-nitrosodiethylamine (NDEA) (Dragan, *et al.,* 1994). Following dietary exposure to

tamoxifen for only three months (40 mg/kg/day) five rats out of a group of fifteen (33%) developed liver tumours, after twenty months of cessation of dosing, with no subsequent promotion (Carthew, *et al.*, 1995a). Promotion with phenobarbital resulted in an earlier development of tumours; where twelve of the fifteen had liver tumours at twenty months. Of the tamoxifen induced adenomas and carcinomas examined, 90% showed depleted nuclear ER expression. Preneoplastic foci were also shown to have a progressively higher incidence of ER depletion as they increased in size, indicating that this effect was associated with promotion of foci to tumours (Carthew, *et al.*, 1997). It is not clear, however, if this down regulation of the ER is related to the promotion process. To establish whether mice develop liver tumours a long term dietary tamoxifen study using three inbred mouse strains was carried out by Martin, *et al.* (1997). It was found that certain strains would not tolerate long-term dosing with tamoxifen (C57B1/6 and DBA/2) due to its oestrogenic effects, which resulted in bone remodelling and kyphosis. The third strain used (B6C3F1), however, survived the full-term of the study (two years) and was found not to develop liver tumours even after phenobarbital was used as a promoter.

No significant increase in uterine tumours was seen following continuous exposure of rats or mice to tamoxifen (Tucker, *at al.*, 1984). In Wistar rats given dietary tamoxifen for three months (420 p.p.m), uteri decreased in size, with an absence of glands in the endometrium, reflecting the action of tamoxifen as an oestrogen antagonist (Carthew, *et al.*, 1996). In B6C3F1 mice dosed with tamoxifen for up to two years there was hyperplasia of the epithelium of the uterine endometrium for the first three months, followed by atrophy of the endometrial component (Martin, *et al.*, 1997). When newborn CD-1 mice were given tamoxifen for the first five days after

birth, 50% of the animals developed uterine adenocarcinomas by the time they reached seventeen months. Control mice did not develop tumours (Newbold, *et al.*, 1997).

4.1.5 Carcinogenicity of tamoxifen in women

Epidemiological data indicated that women taking tamoxifen experience an increased risk of endometrial cancer (Fornander *et al.*, 1989; Blackwood and Weber, 1998). In a study involving >4900 Scandinavian breast cancer patients, there was a 4-fold increase in endometrial cancers during a follow-up time of 8-9 years (Rutqvist *et al.*, 1995). In the National Surgical Adjuvant Breast and Bowel Program (NSABP B-14) in the USA involving 2823 patients with node negative, ER-positive breast cancers, tamoxifen treatment over a 5 year follow-up period resulted in a relative risk (of endometrial cancer) of 7.5 over the placebo group (Fisher, *et al.*, 1994). A case-control study in the USA of the Surveillance, Epidemiology and End Results (SEER) also showed that there was a ~1.6-fold increase in the incidence of uterine tumours in the treated group receiving 20 mg/day tamoxifen (Curtis *et al.*, 1996). In healthy women participating in the Breast Cancer Prevention Trial (BCPT PI) that were treated with tamoxifen, there was a 1.35- to 4.97-fold increased risk of endometrial cancers. In reviewing these data, the IARC concluded that there was sufficient evidence for tamoxifen to be classified as a risk for endometrial cancer (IARC, 1996). The increased risk occurred predominantly in women aged 50 years or older (Fisher *et al.*, 1998). There is a comparatively rapid onset of these tumours within 2-5 years of the start of treatment, suggesting that the mechanism of action may be via an oestrogen agonist effect rather than as a classical chemical carcinogen. There is some weak evidence for an increase in gastrointestinal tract cancers in breast cancer patients

linked to tamoxifen (Rutqvist *et al.*, 1995). This has not been confirmed by other studies (reviewed in IARC, 1996). In the order of 30 lives are saved by tamoxifen treatment to each lost through its side effects. In the case of breast cancer patients, this represents a clear therapeutic advantage (Jordan, 1998).

4.1.6 Genotoxicity of tamoxifen in rodents

Following dosing of rats with tamoxifen, DNA damage was detected in their livers by ^{32}P-postlabelling (Han and Liehr, 1992; White, *et al.*, 1992). These ^{32}P-labelled DNA adducts can be resolved into at least 12 structurally different components when separated by HPLC (Martin, *et al.*, 1998). The extent of DNA damage was related to the time of exposure. In long-term feeding studies where rats were given dietary tamoxifen (40 mg/kg/day) adduct levels increased from approximately 500 adducts/10^8 nucleotides at 30 days to almost 3000 adducts/10^8 nucleotides at 180 days (Carthew, *et al.*, 1995b). Following long-term administration of tamoxifen, DNA adducts were not found in any others tissues, with the exception of the kidney (Li, *et al.*, 1997). Using the more sensitive technique of accelerator mass spectrometry, low levels of irreversible binding to DNA was detected in the livers, reproductive and gastrointestinal tracts of rats after administration of a single dose of ^{14}C-tamoxifen (White, *et al.*, 1997). In the livers of tamoxifen dosed mice the level of DNA adducts was up to 10 fold lower than that detected in rats (White, *et al.*, 1992; Carthew, *et al.*, 1995b; Martin, *et al.*, 1997; Martin, *et al.*, 1998). Following long-term dosing of mice, the level of major DNA adducts did not increase with time of exposure, but after the first month decreased; so much so that by one or two years DNA adduct levels were not significantly above background (Martin, *et al.*, 1997). In rats, loss of DNA adducts after cessation of dosing with tamoxifen takes many weeks (Carthew, *et*

al., 1995a) suggesting that either DNA repair mechanisms are saturated during continuous exposure or that removal of tamoxifen adducts is slow. Recently it has been shown in rat liver that there is a clear dose response relationship between the length of exposure to tamoxifen and both the accumulation of hepatic DNA adducts and lifetime risk of liver cancer (Carthew, *et al.*, 2001). In the absence (but not presence) of phenobarbital promotion there was a threshold value for tamoxifen induced DNA adducts of 128 adducts /10^8 nucleotides and the subsequent induction of liver cancer.

4.1.7 Genotoxicity of tamoxifen in women

The techniques used to detect tamoxifen DNA adducts in rodents have also been used to determine the extent, if any, of tamoxifen induced DNA damage in women undergoing treatment with this drug. Analysis of DNA extracted from the livers of breast cancer patients taking tamoxifen, revealed no tamoxifen specific DNA damage could be detected by ^{32}P-postlabelling. Overall levels of hepatic DNA damage were not significantly different from control individuals not taking the drug (Martin, *et al.*, 1995). In addition, no tamoxifen DNA adducts could be detected, using ^{32}P-postlabelling, in white blood cells obtained from tamoxifen treated women (Phillips, *et al.*, 1996; Bartsch, *et al.*, 2000). However, this is contradicted in a report by Hemminki, *et al.*, (1997) claiming the induction of tamoxifen DNA adducts in the leucocytes of breast cancer patients. There is also some controversial evidence regarding the extent of endometrial DNA damage after tamoxifen administration. A study by Hemminki, *et al.* (1996) detected low levels of approximately 0.27 adducts/ 10^8 nucleotides, considerably lower than the threshold level in rats of 128 adducts /10^8 nucleotides which confers subsequent induction of liver cancer (Carthew, *et al.*,

2001). Another study by Shibutani, et al. (2000a) also detected tamoxifen DNA adducts in 8 out of 16 tamoxifen dosed patients and in none of the control patients (0/15). Levels ranged from 0.2 to 18 adducts/10^8 nucleotides. In contrast, the work of Carmichael, *et al.*, (1996 and 1999) failed to find evidence of tamoxifen DNA adducts in treated patients, using ^{32}P-postlabelling. In all studies, when present, the levels of adducts reported are towards the limit of detection for ^{32}P-postlabelling analysis. Using the more sensitive technique of accelerator mass spectrometry, low levels of irreversible binding to DNA was detected in the endometrium of all ten female volunteers undergoing hysterectomy after administration of a single therapeutic dose of ^{14}C-tamoxifen (20 mg, 50 μCi), a day prior to the operation (Martin, *et al.*, 2001). The mean DNA adduct level of 0.04 adducts/10^8 nucleotides was comparable to levels detected in uterine DNA from rats administered a single equivalent dose of ^{14}C-tamoxifen (0.3 mg/kg). The extent of uterine DNA damage is, however, at least an order of magnitude lower than that found in the livers of similarly treated rats. However, considering the carcinogenic threshold of DNA adducts required in rat liver is 128 adduct/10^8 nucleotides (Carthew, *et al.*, 2001), the significance of this low level of DNA damage in the development of endometrial cancer remains to be determined. If tamoxifen DNA adducts are involved in the development of human endometrial cancer, then perhaps a much lower threshold exists in this tissue compared to rat liver.

4.1.8 Mutagenicity of tamoxifen

Administration of tamoxifen causes GC→TA transversions in both the *cII* and *lacI* genes in lambda/ *lacI* transgenic rats (Davies, *et al.*, 1997 and 1999). However, no mutations were observed in the uterus of rats treated with tamoxifen (Davies, *et al.*, 1997). The pattern of mutations seen in these studies is different from the AT→GC

and GC→AT transitions found in the p53 gene in liver tumours of rats following tamoxifen treatment (Vancutsem, *et al.*, 1994). The presence of transition mutations could, potentially, be explained by the fact that rat liver microsomal activation of tamoxifen and 4-hydroxytamoxifen has been shown to induce production of 8-hydroxy-2'-deoxyguanosine in DNA (Ye and Bodell, 1996). Small adducts such as this could contribute to transition type mutations. Recent studies have shown that site-specific dG-N^2-tamoxifen adducts induce primarily GC→TA transversions in simian kidney (COS-7) cells (Terashima, *et al.*, 1999). Miscoding of damaged DNA consistent with induction of the GC→TA transversion has been demonstrated for the dG-N^2-tamoxifen adduct in site-specifically modified oligonucleotides replicated *in vitro* (Shibutani and Dasaradhi, 1997). Furthermore, using an *in vitro* human nucleotide excision repair system site-specific dG-N^2-tamoxifen adduct isomers in oligodeoxyribonucleotides have been shown to be differentially repaired (Shibutani, et al., 2000b). Overall adducts were removed with a poor to moderate efficiency with the *cis*-forms being removed most efficiently. NMR studies of an 11-mer duplex oligodeoxyribonucleotide containing a *cis*-tamoxifen-N^2-deoxyguanosine adduct have shown that the lesion is accommodated within a widened minor groove without disruption of the surrounding Watson-Crick basepairs (Shimotakahara, *et al.*, 2000). The addition of the tamoxifen DNA adduct does, however, result in bending of the helix by approximately 30°. Although most efforts to date have focused on the major dG-N^2-tamoxifen adducts, it has been previously reported that adducts formed by 4-hydroxytamoxifen are two orders of magnitude more mutagenic in *E-coli* than those arising from α-acetoxytamoxifen (Lowes, *et al.*, 1999).

4.1.9 Aim

To aim of this chapter is to compare the mutagenicity of tamoxifen-DNA adducts replicated in human cells. The *supF* forward mutation assay has been utilised to study mutations induced by α-acetoxytamoxifen, used as a model of sulphated α-hydroxytamoxifen to form α-(deoxyguanosin-N^2-yl)-tamoxifen (Figure 4.4), and 4-hydroxytamoxifen quinone methide (4-OHtamQM), used to form α-(deoxyguanosin-N^2-yl)-4-hydroxytamoxifen (Figure 4.4).

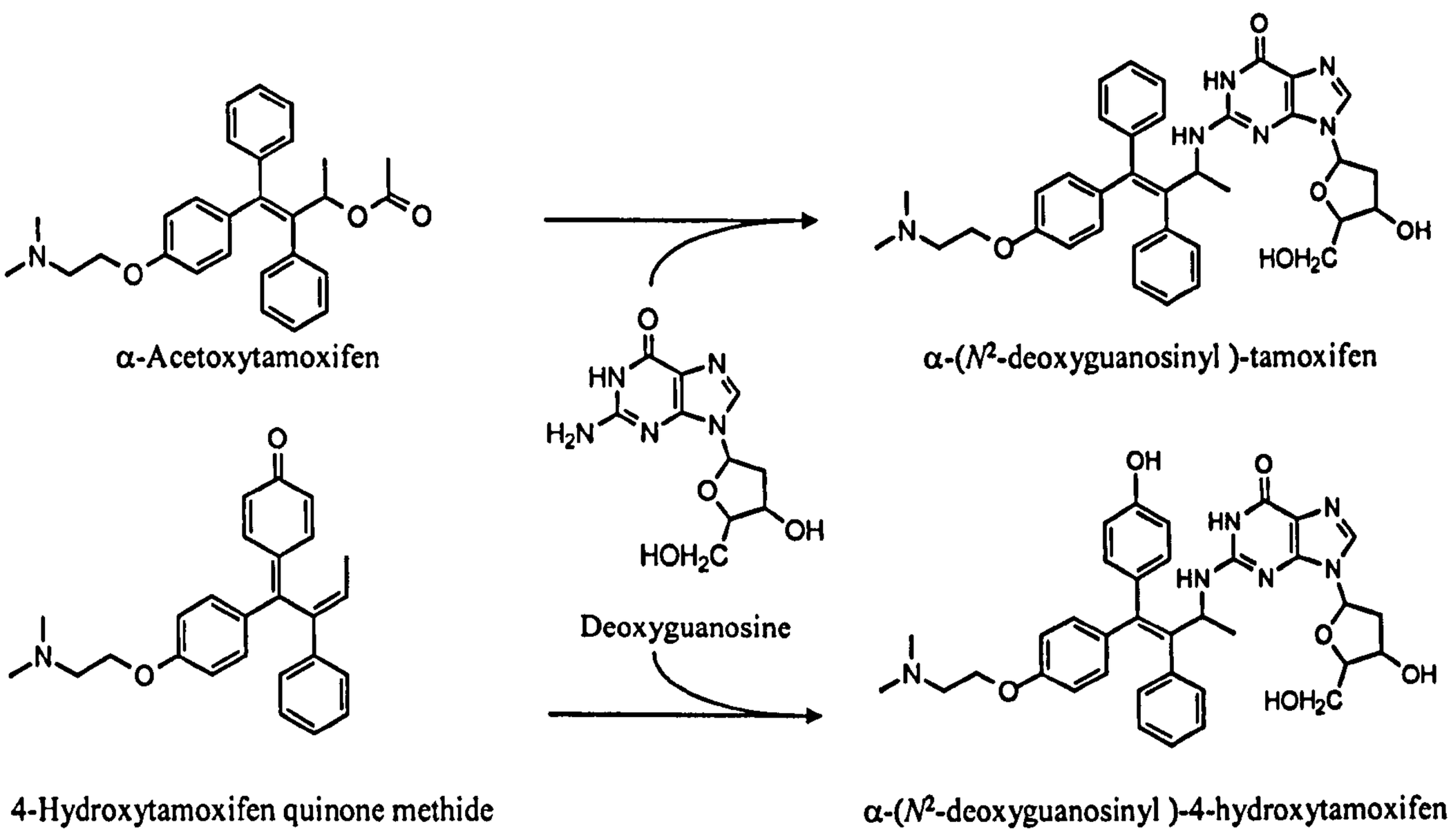


Figure 4.4 The reaction of α-acetoxytamoxifen with deoxyguanosine yields the major α-(deoxyguanosin-N^2-yl)-tamoxifen adduct in rats. Reaction of 4-hydroxytamoxifen quinone methide with deoxyguanosine yields a minor adduct, α-(deoxyguanosin-N^2-yl)-4-hydroxytamoxifen in rats.

The *supF* assay (Seidman, *et al.*, 1985; Parris and Seidman, 1992) has been widely used to study the mutagenicity of compounds that form bulky adducts when reacted

with DNA. The assay detects 97 % of possible base substitutions within the 85 base pair *supF* gene (Routledge, *et al.*, 2001) as well as deletions and insertions, and because the plasmid is treated *in vitro*, aliquots of the treated DNA can be analysed for adduct quantification in parallel to the mutation assay. To this end, a previously developed ^{32}P-postlabelling method for analysis of tamoxifen DNA adducts was used (Martin, *et al.*, 1998).

4.2 Materials and Methods

4.2.1 Materials

All chemicals were from Sigma-Aldrich, Poole, Dorset, UK unless otherwise stated.

4.2.1.1 Human cell lines

Human embryonic adenovirus-transformed kidney cells (Ad293) were cultured from cells provided by Dr. A. Dipple, National Cancer Institute, Frederick, MD, USA. Ad293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Life Technologies Ltd, Paisley, UK) at 37°C in 5% CO_2 in air. Human SV40 transformed *Xeroderma pigmentosum* complementation group A fibroblasts (GM04429) and human SV40 transformed apparently normal non-foetal cells (GM00637) were obtained from NIGMS Human Genetic Cell Repository, Camden, NJ, USA. Cells were grown in Dulbecco's modified Eagle's medium (with Earle's salts) supplemented with 10% (GM04429) or 15% (GM00637) fetal calf serum (Life Technologies Ltd, Paisley, UK) at 37°C in 5% CO_2 in air.

Methods

All methods specific to this chapter are described below. For any other procedures please refer to chapter 2.

4.2.2 Acetylation of α-hydroxytamoxifen

The *trans* isomer of α-hydroxytamoxifen was synthesised using the method described by Foster and co-workers (Foster, *et al.*, 1985) by Dr. Karen Brown. *trans* α-

Acetoxytamoxifen was prepared from *trans* α-hydroxytamoxifen using the published method (Osborne, *et al.*, 1996). α-Hydroxytamoxifen (100 mg, 260 μmol) was washed with 3×5 ml pyridine (followed by rotary evaporation each time). α-Hydroxytamoxifen was redissolved in anhydrous pyridine (2 ml) and anhydrous acetic anhydride (100 μl, 100 μmol) was added. After 18 h at 37°C the mixture was diluted with diethyl ether (50 ml) and washed with water (3×30 ml). The organic layer was dried (Na_2SO_4) and concentrated. The product, α-acetoxytamoxifen was separated from unreacted starting materials by column chromatography (2% methanol in dichloromethane). Mass analysis of the product revealed a protonated molecular ion ($M+H^+$) with m/z 430 confirming the molecular formula $C_{28}H_{31}NO_3$ (429.230). Purified α-acetoxytamoxifen was dried by rotary evaporation, and the residue re-dissolved in ethanol.

4.2.3 Treatment of pSP189 plasmid with α-acetoxytamoxifen

Aliquots of pSP189 plasmid (200 μg, in 200 μl tris-EDTA buffer, pH 8.0) were treated with varying doses of α-acetoxytamoxifen in 100 μl ethanol (10 μM, 25 μM and 50 μM final concentrations). Treated plasmid, in triplicate, along with a single ethanol treated control (200 μg plasmid only) was incubated overnight at 37°C. Unreacted compound was extracted from the treatment mixture with water saturated ethyl acetate (3 × 400 μl), using a needle and syringe to remove the organic layer. The DNA was precipitated with 2 M sodium acetate (10 μl) and ice-cold ethanol (800 μl). The DNA samples were pelleted by centrifugation using a bench top centrifuge (Hettich, Germany), supernatants removed and pellets washed with 70% ice-cold ethanol followed by 100% ice-cold ethanol. Pellets were re-dissolved in 400 μl sterile

tissue culture grade water. The yield of DNA was calculated from the optical density at 260 nm.

DNA concentration = A_{260} × Dilution × 50 (constant for dsDNA)

The plasmid DNA was stored at -80°C until transfection into cells was performed.

4.2.4 Treatment of pSP189 plasmid with 4-OHtamQM

4-Hydroxytamoxifen (6.52 mg) was activated to its quinone methide by stirring for 30 min with silver (II) oxide (42.92 mg) in 1.8 ml dry chloroform (Liehr, *et al.*, 1983). The reaction mixture was filtered and dried to a yellow-brown residue under nitrogen. Assuming 100% conversion, this was dissolved in 50:50 ethanol: acetonitrile, and added to 100 μg pSP189 plasmid in 500 μl H_2O to give a total volume of 625 μl. Final concentrations of 0, 50, 100 and 250 μM were used. After incubation at 37°C for 18 h the unreacted 4-OHtamQM was extracted from the plasmid with 5× 400 μl diethyl ether. Plasmid DNA was precipitated with 3M sodium acetate / ice-cold ethanol and re-dissolved in 200 μl sterile tissue culture grade water. The yield of DNA was calculated from the optical density at 260 nm. The plasmid DNA was stored at -80°C until transfection into cells was performed.

4.2.5 ^{32}P-Postlabelling of α-acetoxytamoxifen and 4-OHtamQM treated pSP189 plasmid DNA

4.2.5.1 Digestion

The method is outlined in Chapter 1, Figure 1.8. α-Acetoxytamoxifen or 4-OHtamQM treated pSP189 plasmid, control plasmid and liver DNA from a tamoxifen treated rat (positive control) (5 μg of each) were dried by vacuum centrifugation in

1.5 ml ultra-centrifuge tubes. To each tube was added 6.25 µl digestion mixture. The digestion mixture contained 175 mU micrococcal nuclease, 3 mU calf spleen phosphodiesterase, 1 µl SSCC buffer (100 mM sodium succinate and 50 mM calcium chloride) and sterile water to make up to 6.25 µl. Samples were incubated overnight (17 h) at 37°C.

4.2.5.2 Nuclease P1 treatment

After overnight digestion 5 µl (4 µg) was removed from the digestion mixture and transferred to a separate tube. The remaining 1µg was retained for later use (Section 4.2.5.4). To enrich the samples for adducts nuclease P1 (9 µg) was added to each tube and incubated at 37°C for 1 hour. Tris HCl (10 mM, pH 7.6, 2.4 µl) was added to stop the incubation.

4.2.5.3 Radiolabelling of adducted nucleotides

T4 polynucleotide kinase (Roche Diagnostics Ltd., Lewes, East Sussex, UK) (0.5 µl) and labelling buffer (2 µl) (containing 200 mM Tris HCl, 100 mM magnesium chloride, 100 mM DTT, 10 mM spermidine, filter sterilised) was added to each nuclease P1 treated sample. The samples were transferred to a perspex box and ^{32}P-radiolabelled ATP (50µCi [γ-^{32}P]ATP, Amersham Life Sciences Ltd, Little Chalfont, Bucks., UK) was added to each sample (volume was dependent on the specific activity (SA)). The samples were incubated at 37°C for 1 hour then an aliquot from each tube was removed and diluted (1:20). This was used to analyse the efficiency of

the nuclease P1 enrichment step. The remaining samples were stored at -20°C until HPLC analysis.

4.2.5.4 Labelling normal nucleotide samples

The 1μg samples remaining after the digestion step (Section 4.2.5.1) were diluted by a factor of 4800 and postlabelled as above (Section 4.2.5.3) with 20 μCi [γ-^{32}P] ATP. The samples were split into two aliquots: one aliquot was incubated with apyrase (80 mU) for 30 minutes at 37°C, (+apyrase) whilst the other was incubated without enzyme (-apyrase). The aliquots were diluted with sterile water (final volume 100 μl).

4.2.5.5 Efficiency of labelling and nuclease P1 digestion

+Apyrase, –apyrase and nuclease P1 efficiency samples were spotted (2 μl) onto polyethyleneimine (PEI)-cellulose thin layer chromatography (TLC) plates (Merck Eurolab Ltd., Poole, Dorset UK), and developed in 0.12 M sodium phosphate, pH 6.8 for approximately 2 hours. The plates were dried, wrapped in Saran wrap and scanned using a phosphorimager.

4.2.6 HPLC analysis of ^{32}P-postlabelled α-acetoxytamoxifen or 4-OHtamQM treated pSP189 plasmid DNA

4.2.6.1 HPLC instrumentation

Samples were injected manually (0.5µl or 1µl DNA in 100µl water) onto a Varian Prospekt solvent delivery unit (Varian Ltd., Walton on Thames, Surrey, UK) and the adducted ^{32}P-labelled nucleotides were eluted onto a HPLC column (Hypersil C18, 5 µm, 250x4.6 mm reversed-phase column; Phenomenex, Macclesfield, Cheshire, UK). The HPLC pump was a Varian Star 9012. Eluate passed through a UV-Vis detector (Varian Star 9050) and a radiochemical detector (β-ram, Lablogic, Sheffield, UK) with a solid phase cell (500 µl). Data analysis was by Laura, an MS Windows package (Lablogic).

4.2.6.2 HPLC conditions

HPLC analysis was performed at ambient temperature. Solvent A was 2 M ammonium formate pH 4.0. This was prepared by dissolving ammonium formate in water and adjusting the pH to 4.0 with 98% formic acid. Solvent B was a mixture of acetonitrile and methanol 6:1 (v/v). Optimum separation was achieved with 1 ml/min of 80% A, 20% B for 40 minutes followed by a linear gradient of 20-45% B for 20 minutes.

4.2.6.3 HPLC adduct calculations

Known amounts of ATP were injected onto the HPLC, a peak (eluting at 3 min) was collected and radioactivity measured in disintegrations per min (d.p.m.) by

scintillation counting. The ATP peak area was plotted against d.p.m. to give a standard curve. HPLC peak areas were measured and values applied to this standard curve to give values in d.p.m. Relative adduct labelling (RAL) was then calculated by the method of Reddy and Randerath (1986) based on the specific activity (SA) of the [γ-^{32}P]ATP (expressed as d.p.m. per pmol) and the amount of DNA used (1μg DNA = 3240 pmol deoxyribonucleoside 3'-monophosphates [dNp])

RAL = d.p.m. in adduct peak / (SA ATP × pmol dNp used for analysis)

4.2.7 Fugene-6 mediated transfection of Ad293 cells with α-acetoxytamoxifen and 4-OHtamQM treated pSP189 plasmid DNA

Transfection was carried out as described previously in Chapter 3.

4.2.8 Fugene-6 mediated transfection of GM00637 and GM04429 cells with α-acetoxytamoxifen treated pSP189 plasmid DNA

GM00637 or GM04429 cells (grown at 37°C, 5% CO_2, 90% confluent) were split 1 in 10 (approximately 2×10^6 cells) and plated in 9 cm transfection plates in 10 ml Dulbecco's modified Eagle's medium with Earle's salts (Life Technologies Ltd., Paisley, UK) (10-15% foetal calf serum) and grown for 68 h (37°C, 5% CO_2). Cells were 40-60% confluent prior to transfection. Three hours before transfection, used medium was aspirated and 10 ml fresh medium added. Fugene-6 (Roche Diagnostics Ltd, Lewes, East Sussex, UK) transfection reagent (15 μl) was slowly added to foetal

calf serum free Dulbecco's modified Eagle's medium (500 µl). The tube was gently tapped to mix contents. Plasmid DNA (10 µg) was added to the Fugene solution and incubated at room temperature for 20 min. The aliquot of DNA was carefully added drop-wise to each transfection plate and swirled gently to mix. Plates were returned to the incubator (37°C, 5% CO_2). Twenty-four hours after transfection used media was aspirated and replaced with 10 ml fresh media. The plasmid was reclaimed from the GM04429 cells between 45-48 h after transfection, and reclaimed from the GM00637 cells 60 h after transfection as described in Chapter 2. GM00637 cells had a longer doubling time, so differential growth time yielded approximately the same amount of cells (~10-12×10^6).

4.2.9 Isolation of pSP189 plasmid, transformation of E.coli MBM7070, and screening for mutant colonies

Plasmid was recovered and treated with Dpn1 restriction enzyme as described previously in Chapters 2 and 3. Transformation and screening of *E.coli* MBM7070 was carried out as described previously in Chapter 2.

4.3 Results

The mutagenicity of the major tamoxifen DNA adduct, induced by treatment with α-acetoxytamoxifen, and one of the minor tamoxifen DNA adducts, induced by treatment with 4-OHtamQM, was compared using the *supF* forward mutation assay and human adenovirus transformed kidney (Ad293) cells. Studies were also performed to investigate the role of nucleotide excision repair in the induction of mutations by α-acetoxytamoxifen using the *supF* forward mutation assay. Plasmid was replicated in cells which were either repair proficient (GM00637) or repair deficient (GM04429). Human SV40 transformed GM00637 cells are derived from an apparently normal 18 year old Puerto Rican Caucasian female. Human SV40 transformed GM04429 cells (also known as XP12BE) are derived from a 7 year old Caucasian female suffering from the genetic disease *Xeroderma pigmentosum* (see Chapter 1). This disease has been further sub-divided, and she is in complementation group A, meaning that she lacks the *XPA* gene. As a result of this disease these cells are deficient in nucleotide excision repair (see Chapter 1).

Treated plasmid was transfected into either, Ad293, GM00637 or GM04429 cells where replication took place. Recovered plasmid was used to transform MBM7070 indicator *E. coli*. Any plasmids containing a mutation in the *supF* gene grew as white colonies whilst non-mutant wild type plasmids grew as blue colonies. Mutant colonies were grown to amplify the plasmid and were sequenced to determine the mutation(s) in the *supF* gene region of the plasmid.

4.3.1 Tamoxifen-DNA adduct quantification by ^{32}P-postlabelling

4.3.1.1 DNA adduct quantification by ^{32}P-postlabelling of α-acetoxytamoxifen treated pSP189 plasmid

Shuttle vector plasmid pSP189 was modified by reaction with α-acetoxytamoxifen and analysed by ^{32}P-postlabelling to quantify adduct levels at different doses. A positive control of liver DNA from a Wistar Han rat which had been treated with tamoxifen in the diet for six months (40 mg/kg) was also analysed in parallel. Figure 4.5(b) shows that as reported previously (Martin, *et al.*, 1998), α-acetoxytamoxifen treated plasmid gave one major peak that co-eluted with the dG-N^2-tamoxifen adduct detected in DNA from tamoxifen treated rats (Figure 4.5d). An additional minor peak is also observed eluting just prior to the major adduct, which, based on retention time, is the *N*-demethylated dG-N^2-tamoxifen adduct (Brown, *et al.*, 1999). The degree of pSP189 modification by α-acetoxytamoxifen equates to a level of 0.5, 1.6 or 2.5 adducts per plasmid (4952 basepairs), for the 10, 25 and 50 μM doses respectively (Table 4.1), which is equivalent to a range of between 50-240 adducts per 10^6 nucleotides.

The same batch of plasmid was treated with α-acetoxytamoxifen and then used for all *supF* assay investigations in Ad293 cells, GM00637 normal cells and GM04429 XP cells. Raw α-acetoxytamoxifen postlabelling data is tabulated in Appendix 2.

4.3.1.2 DNA adduct quantification by ^{32}P-postlabelling of 4-OHtamQM treated pSP189 plasmid

Shuttle vector plasmid pSP189 was modified by reaction with 4-OHtamQM and analysed by ^{32}P-postlabelling to quantify adduct levels at different doses. A positive control of liver DNA from a Wistar Han rat which had been treated with tamoxifen in the diet for six months was used (40 mg/kg). Following incubation of 4-OHtamQM with plasmid DNA, two main ^{32}P-postlabelled adduct peaks are detected (Figure 4.5c).

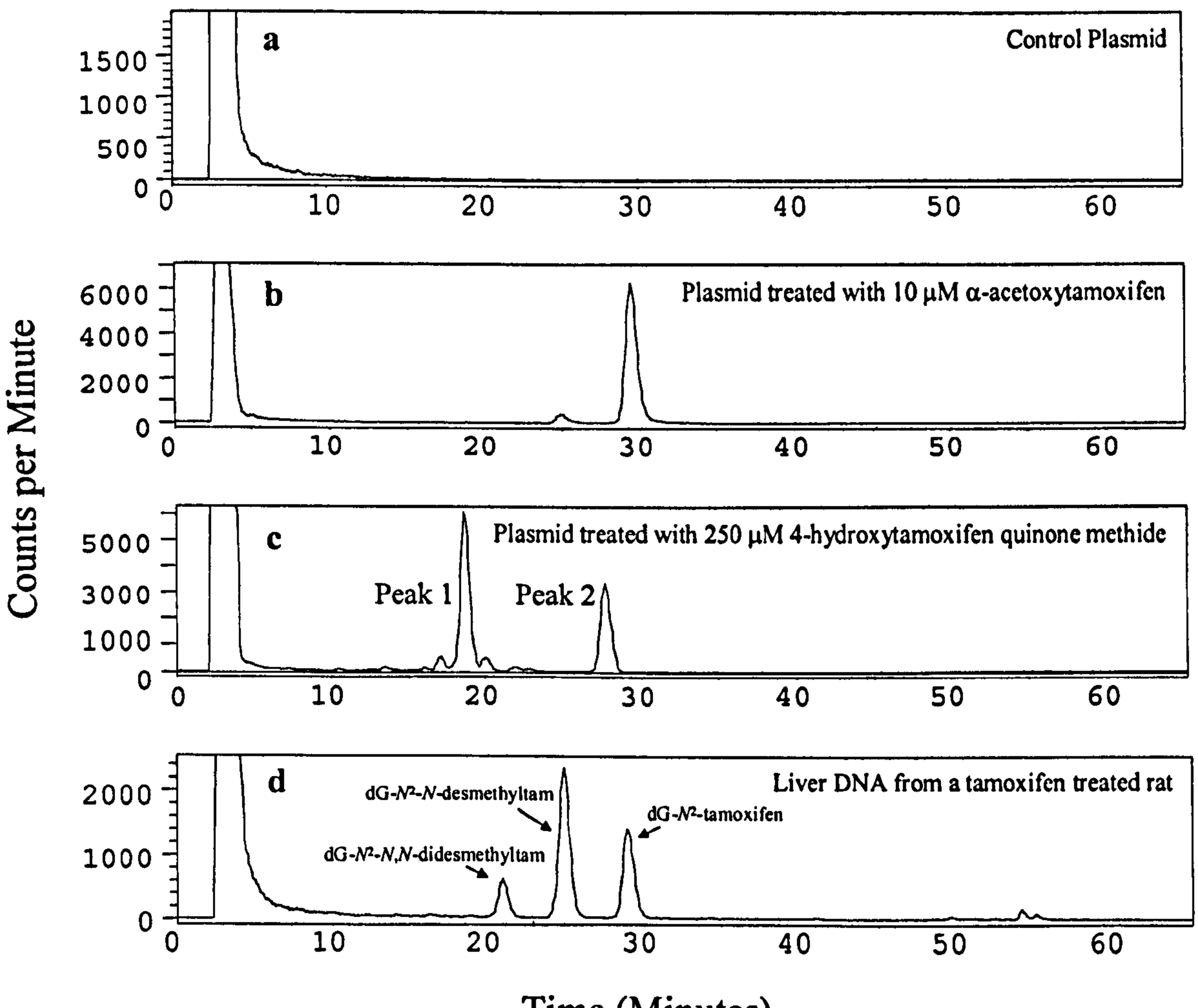


Figure 4.5 Representative radioactive HPLC chromatograms of ^{32}P-labelled digests from control, *a*-acetoxytamoxifen and 4-OHtamQM treated plasmids, and liver DNA from a tamoxifen treated Wistar Han Rat (40 mg/kg tamoxifen in diet for 6 months)

Although these peaks do not co-elute with any adduct peaks detected in DNA from rat liver tissue shown in Figure 4.5d, it has been previously demonstrated the presence of up to twelve adduct peaks in similarly treated rats and one of these co-elutes with the main 4-hydroxytamoxifen derived adduct peak (Martin, *et al.*, 1998). The major products of the reaction of 4-OHtamQM, produced by chemical oxidation, with DNA are known to be isomers of a 4-hydroxylated form of dG-N^2-tamoxifen (α-(deoxyguanosin-N^2-yl)-4-hydroxytamoxifen) (Marques and Beland, 1997). This reactive intermediate can also be generated enzymatically and it has been shown that incubation of 4-hydroxytamoxifen with horseradish peroxidase yields one major ^{32}P-postlabelled adduct peak, which corresponds to peak 1 observed in this study (Martin, *et al.*, 1998; Lowes, *et al.*, 1999).

This difference in adduct profile is probably due to the different methods used to activate 4-hydroxytamoxifen. The additional adduct, which is present at low levels with peroxidase activation, may be an isomer of dG-N^2-4-hydroxytam or may be an as yet unidentified adduct. Higher concentrations of 4-OHtamQM were used in the incubations compared to α-acetoxytamoxifen, as the former is known to generate lower levels of adducts (Lowes, *et al.*, 1999). In the present study, 4-OHtamQM (50 μM) induced a 50 fold lower level of DNA adducts than an equimolar dose of α-acetoxytamoxifen. The three treatments with 4-OHtamQM (50, 100 and 250 μM) induced approximately 0.05, 0.1 and 0.2 adducts per plasmid (Table 4.5), which is equivalent to a range of between 5-20 adducts per 10^6 nucleotides. Raw 4-OHtamQM postlabelling data is tabulated in Appendix 3.

4.3.2 Part A: Mutagenicity of plasmid treated with α-acetoxytamoxifen replicated in Ad293 cells

4.3.2.1 Mutation frequency in *supF* gene

Treatment of pSP189 plasmid with α-acetoxytamoxifen induced an increase in mutation frequency for all doses in comparison to solvent control (ethanol), as illustrated in Table 4.1. Treatment with the two lower doses of α-acetoxytamoxifen induced a low mutation frequency increase relative to the control (1.6-fold for 10 μM, and 1.4-fold for 25 μM). The highest dose (50 μM) increased mutation frequency by 4.9-fold over control. Interestingly the mutation frequency of plasmid treated with water only was 0.2×10^{-4}, 33-fold lower than ethanol treated control.

Table 4.1. Mutation frequency induced by α-acetoxytamoxifen replicated in Ad293 cells

Treatment	Colonies Screened	Number of Mutants	Mutation Frequency[a]	Adduct Number[b] (± S.D.)
Solvent Control[c]	33000	22	6.6	0 ± 0
10 μM	34000	36	10.5	50 ± 8.8
25 μM	21500	20	9.3	160 ± 8
50 μM	28000	92	32.3	240 ± 52

[a] Mutation frequency per 10^4 colonies

[b] Adduct number per 10^6 nucleotides

[c] Mutation frequency of plasmid dissolved in water only was 0.2×10^{-4}

4.3.2.2 Mutation types found in *supF* gene

The types of mutations induced by α-acetoxytamoxifen are illustrated in Table 4.2. For all sequenced plasmids, with or without α-acetoxytamoxifen treatment, the predominant mutations are in the form of base substitutions (ranging from 75% to

94% of all mutations seen). When these are subdivided into single base, tandem or multiple mutations, the single base substitutions are most common (between 60% and 76%). Tandem substitutions range from 0% to 9% and multiple mutations accounted for between 5% and 19%. The proportion of frameshift mutations ranges from 5.6% to 25%.

Table 4.2. Types of sequence alterations in *supF* gene of pSP189 plasmids treated with α-acetoxytamoxifen replicated in Ad293 cells

Mutations	Number of plasmids with mutations (%)			
	Control	10 μM	25 μM	50 μM
Base substitutions	**19** (86)	**34** (94)	**15** (75)	**83** (90)
Single	14 (64)	27 (75)	12 (60)	70 (76)
Tandem	2* (9.1)	0 (0)	2 (1.5)	1 (1.1)
Multiple	3 (14)	7 (19)	1 (5)	12 (13)
Frameshifts	**3** (14)	**2** (5.6)	**5** (25)	**9** (9.8)
Single base deletion	1 (4.5)	0 (0)	1 (5)	2 (2.2)
>2 bases deletion	2 (9.1)	2 (5.6)	4 (20)	7 (7.6)
Single base insertion	0 (0)	0 (0)	0 (0)	0 (0)
>2 bases insertion	0 (0)	0 (0)	0 (0)	0 (0)
Total plasmids sequenced	**22** (100)	**36** (100)	**20** (100)	**92** (100)

* three adjacent bases substituted

Table 4.3 shows the types of single base substitutions induced by α-acetoxytamoxifen. At all dose levels, including control, transversions are the preferred mutation (59-88% of all substitutions). In the ethanol treated control plasmid the major mutations are GC→TA transversions and GC→AT transitions (each accounting for 37% of all substitutions) with GC→CG transversions contributing 21% and AT→TA transversions 5% of base substitutions.

Table 4.3. Types of single and tandem base substitution mutations in *supF* gene of pSP189 plasmid treated with α-acetoxytamoxifen replicated in Ad293 cells

Mutations	Number of plasmids with mutations (%)			
	Control	10 μM	25 μM	50 μM
Transversions	**12** (63)	**16** (59)	**14** (88)	**53** (74)
GC→TA	7 (37)	6 (22)	10 (63)	44 (61)
GC→CG	4 (21)	8 (30)	3 (19)	3 (4)
AT→TA	1 (5)	0 (0)	1 (6.3)	5 (6.9)
AT→CG	0 (0)	2 (7.4)	0 (0)	1 (1.4)
Transitions	**7** (37)	**11** (41)	**2** (13)	**19** (26)
GC→AT	7 (37)	9 (33)	1 (6.3)	17 (24)
AT→GC	0 (0)	2 (7.4)	1 (6.3)	2 (3)
Total single base substitutions	**19** (100)	**27** (100)	**16** (100)	**72** (100)

At the 10 μM α-acetoxytamoxifen dose the major mutation is the GC→AT transition (33%), followed by GC→CG (30%), GC→TA (22%) transversions with AT→CG and AT→GC accounting for around 7% each. At the two higher doses there is a pronounced preference for the induction of GC→TA transversions (63% at 25 μM and 61% at 50 μM). At the 25 μM dose the other major substitution is the GC→CG transversion with AT→TA transversions and GC→AT and AT→GC transitions being relatively uncommon (6% each). After treatment with the highest dose (50 μM) the second most common substitution is the GC→AT transition (24%), followed by AT→TA transversion (7%), GC→CG transversion (4%), and AT→GC transition (3%).

Figure 4.6 shows a comparison of the different proportions of base substitution mutations induced in the *supF* gene by the various α-acetoxytamoxifen treatments or in the ethanol treated control. As the dose increases, the proportion of GC→TA transversions generally increases and the proportion of GC→AT transitions decreases. It is clear from the graphical representation that transversions are the major substitution types in both the control and treated samples.

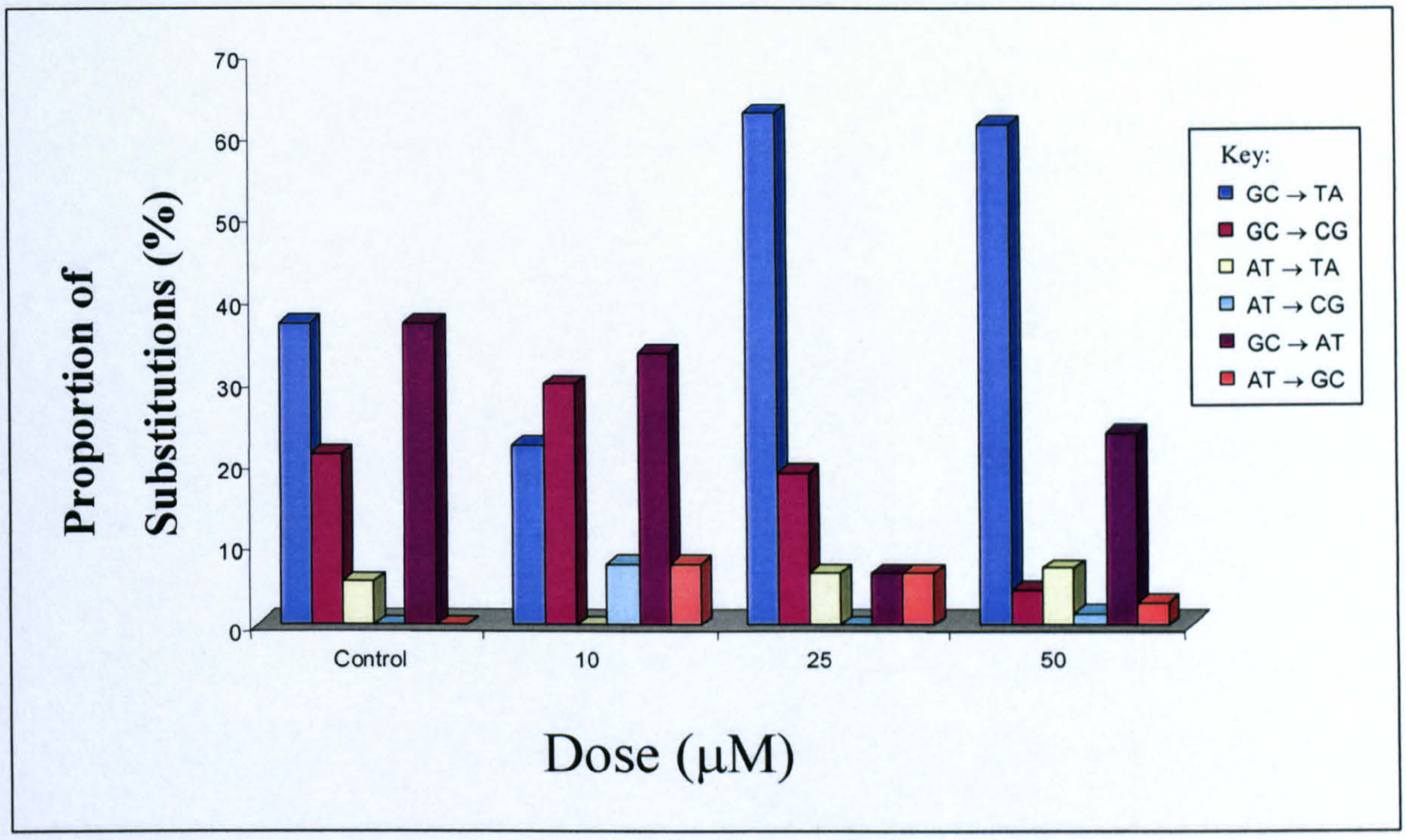


Figure 4.6 Bar chart illustrating the proportion of the different single base and tandem substitution mutations seen in ethanol treated control mutants and in those induced by treatment with α-acetoxytamoxifen (10 μM, 25 μM and 50 μM).

Transversions are also preferentially induced in plasmids with multiple mutations after treatment with α-acetoxytamoxifen (Table 4.4). In the control plasmid sample there was no overall preference for either transversions or transitions. GC→AT transitions accounted for 44% of all control multiple mutations, with GC→TA (22%), GC→CG (11%), AT→TA (11), AT→CG (6%) transversions accounting for 22%, 11%, 11% and 6% respectively and AT→GC transitions for 6%.

Table 4.4. Types of multiple base substitution mutations in *supF* gene of pSP189 plasmids treated with α-acetoxytamoxifen replicated in Ad293 cells

Mutations	Number of plasmids with mutations (%)			
	Control	10 μM	25 μM	50 μM
Transversions	**9** (50)	**20** (63)	**3** (75)	**38** (55)
GC→TA	4 (22)	4 (13)	0 (0)	26 (38)
GC→CG	2 (11)	6 (19)	1 (25)	8 (12)
AT→TA	2 (11)	6 (19)	1 (25)	3 (4.3)
AT→CG	1 (6)	4 (13)	1 (25)	1 (1.4)
Transitions	**9** (50)	**12** (38)	**1** (25)	**31** (45)
GC→AT	8 (44)	9 (28)	1 (25)	30 (43)
AT→GC	1 (5.6)	3 (9.4)	0 (0)	1 (1)
Total multiple base substitutions	**18** (100)	**32** (100)	**4** (100)	**69** (100)

After treatment with 10 μM α-acetoxytamoxifen, the major multiple substitution is the GC→AT transition (28% of all mutations), GC→CG and AT→TA transversions account for 19% each, GC→TA and AT→CG transversions for 13% each and AT→GC transitions for 9.4%. Dosing the plasmid with 25 μM α-acetoxytamoxifen only yielded one multiple mutation. This had four substitutions, one each of GC→CG, AT→TA, and AT→CG transversions and a GC→AT transition. The 50 μM treatment induced multiple substitutions preferentially in the form of GC→AT transitions (43%), closely followed in magnitude by GC→TA transitions (38%). The remaining substitutions were all 4% or less, with the exception of GC→CG transversions (12%).

Figure 4.7 illustrates the comparison of the different proportions of base substitution mutations induced in the *supF* gene by the various α-acetoxytamoxifen treatments, and through spontaneous means. Looking at the bar charts, there are no clear trends. However, transversions are more prevalent in all treatments (including control) but in all cases (except at 25 μM due to the low amount of data) the major substitution is the GC→AT transition.

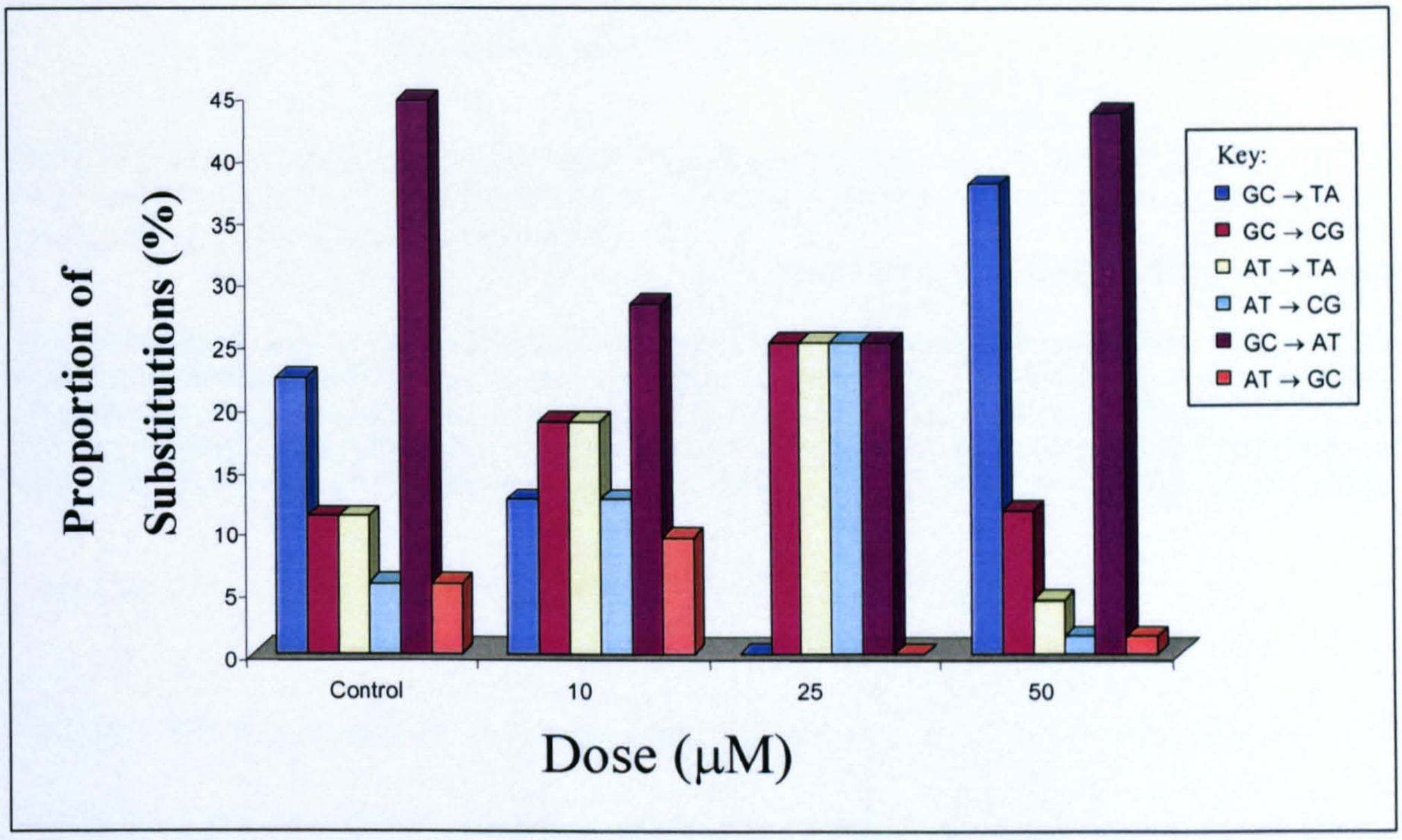


Figure 4.7 Bar chart illustrating the proportion of the different multiple base substitution mutations seen in ethanol treated control mutants and in those induced by treatment with α-acetoxytamoxifen (10 μM, 25 μM and 50 μM).

The proportion of frameshift mutations seems to vary with dose, compared to control. Of the control plasmids sequenced 14% had deletions (no insertions were seen at any dose), whereas at 10 μM, 25 μM and 50 μM there were deletions in the proportions 5.6%, 25% and 9.8%, respectively. Of these deletions there was a preference for larger deletions at all doses including control. The deleted sequences are illustrated in Figure 4.8.

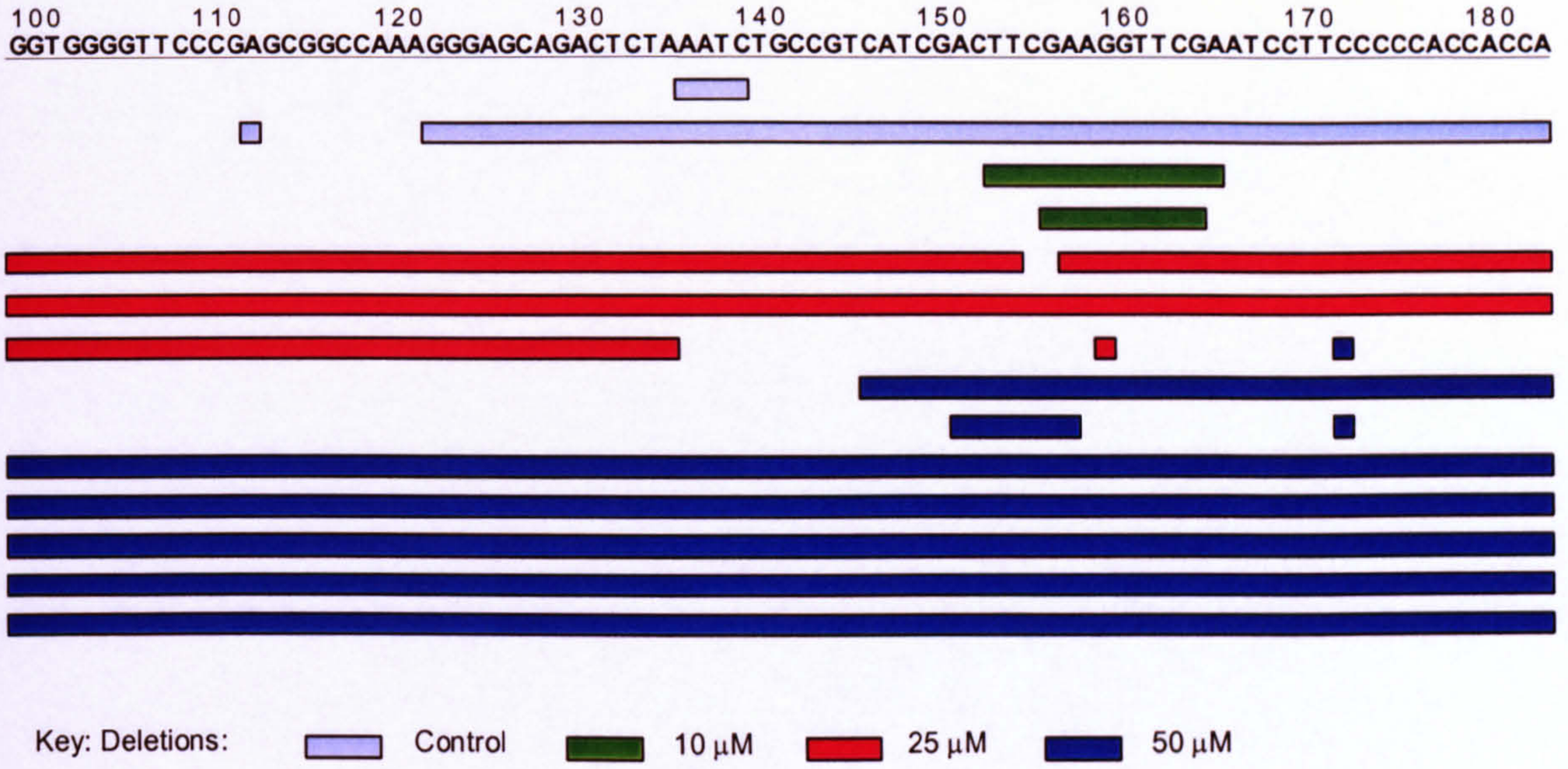


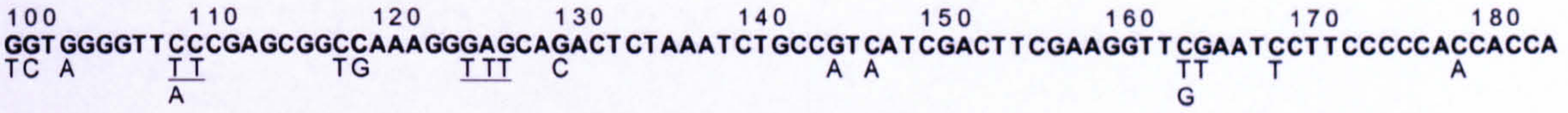
Figure 4.8 Deletions seen in spontaneous mutants and in those induced by treatment with α-acetoxytamoxifen (10 μM, 25 μM and 50 μM).

4.3.2.3 Mutation spectra in *supF* gene

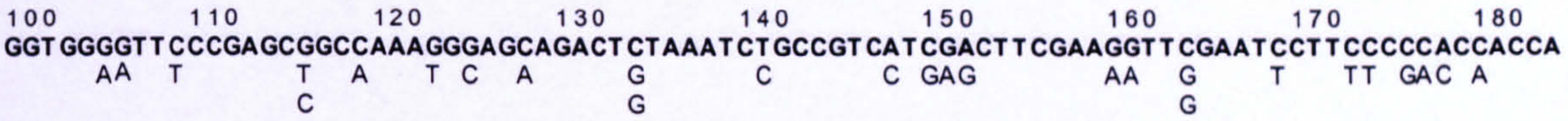
The distribution of single base and tandem substitution mutations within the *supF* gene for plasmids dosed with α-acetoxytamoxifen (10 μM, 25 μM and 50 μM) or control treatments is shown in Figure 4.9. When these spectra are compared using the hyperg program (Cariello, *et al.,* 1994), treated spectra are found to be significantly different from control (p (same) ≤ 0.05). Mutations are not distributed randomly, but concentrated at one or more sites, known as hotspots.

Single and multiple base substitutions have been presented on separate spectra because it has been suggested that these multiple mutations arise through a different mechanism to single base mutations (Courtemanche and Anderson, 1999).

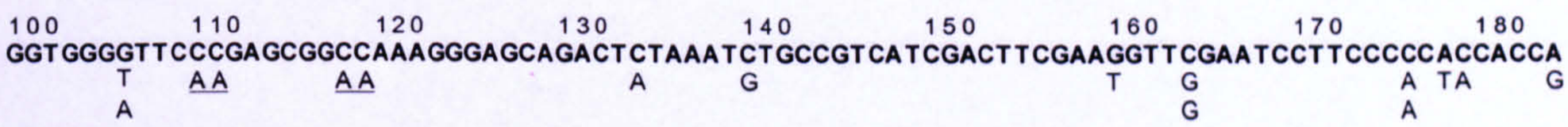
Non-treated Control



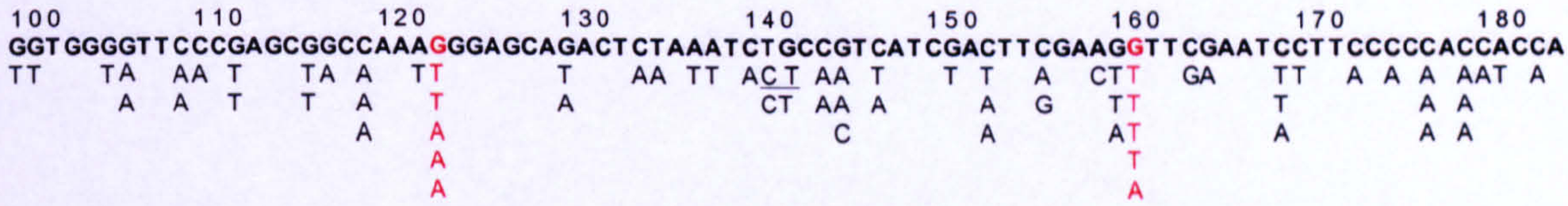
10 μM α-Acetoxytamoxifen



25 μM α-Acetoxytamoxifen



50 μM α-Acetoxytamoxifen



α-Acetoxytamoxifen combined

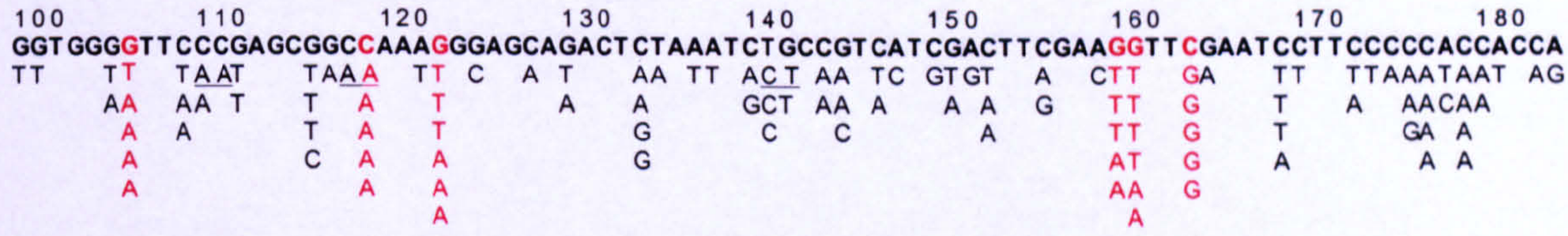


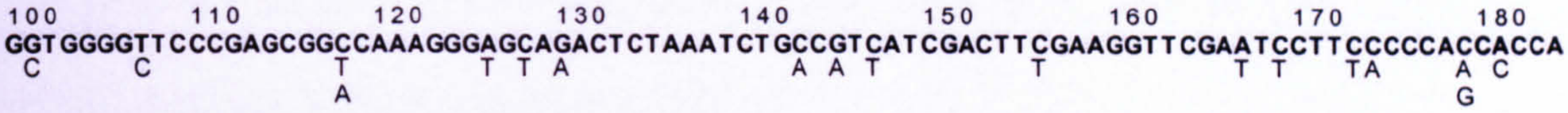
Figure 4.9 Mutation spectra depicting single base and tandem substitutions induced after treatment with α-acetoxytamoxifen or in ethanol treated control (10 μM, 25 μM and 50 μM). Hotspots are shown in red.

The mutation spectra generated from single and tandem base substitution mutations induced spontaneously and by treatment with α-acetoxytamoxifen is illustrated in Figure 4.9. In the mutation spectra induced by control treatment with solvent only or at the two lower doses (10 μM and 25 μM) there are no hotspots. At the higher dose

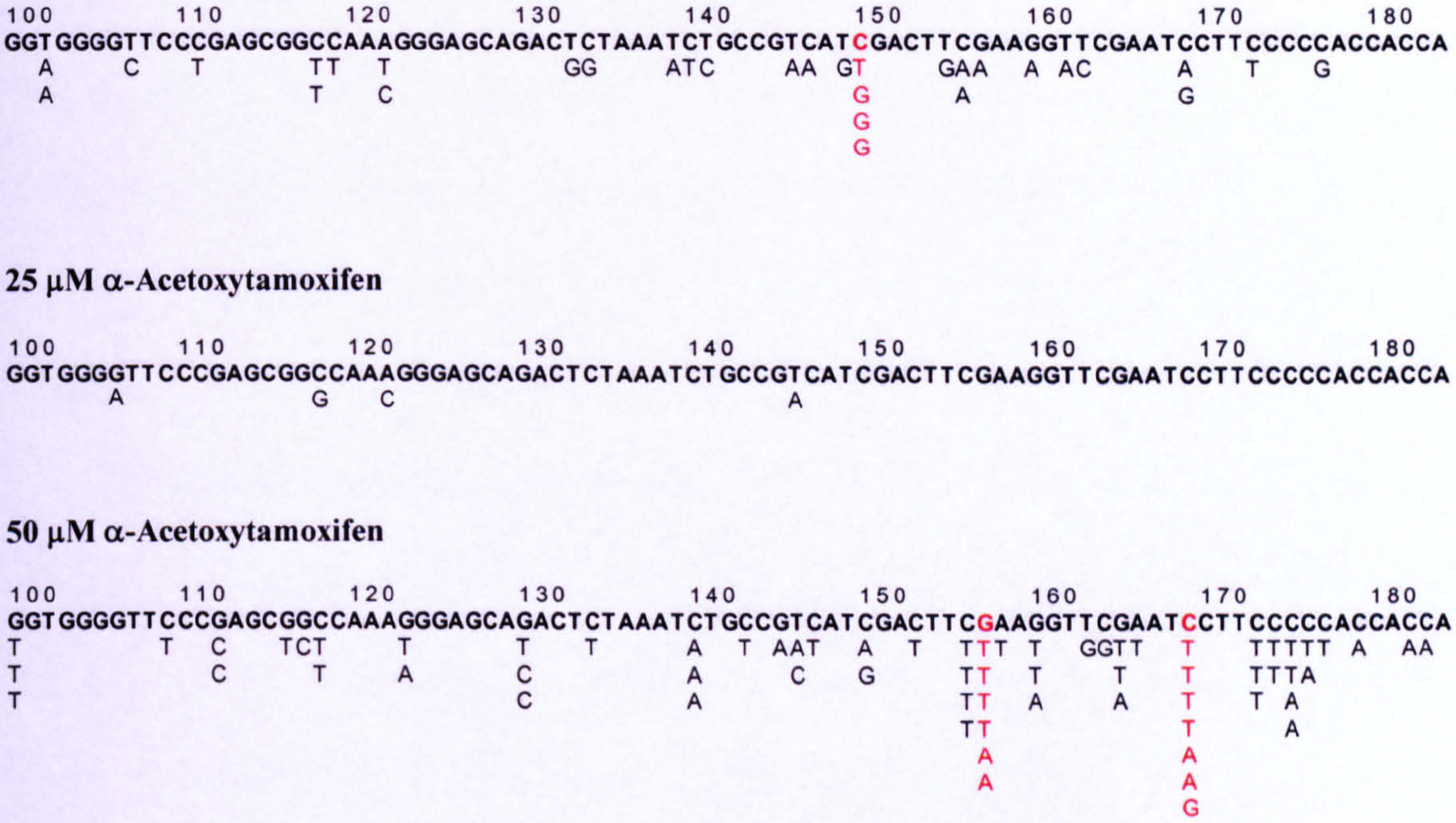
(50 μM) there are two hotspots at positions 122 and 160. If all three mutation spectra from α-acetoxytamoxifen treated plasmids are combined there are now six hotspots at positions 105, 118, 122, 159, 160 and 163. Positions 122 and 160 are sites of hotspots in both the 50 μM and combined mutation spectra. All hotspots from treated spectra are at positions of GC base pairs.

In the mutation spectra illustrating multiple base substitution mutations (Figure 4.10) there are both similarities and differences compared to Figure 4.9. In the mutation spectrum from control treated plasmid no hotspots are seen. After treatment with 10 μM α-acetoxytamoxifen, a single mutation hotspot is induced at the GC basepair of position 149. There were not enough multiple substitutions to give a mutation spectrum for the dose of 25 μM α-acetoxytamoxifen but at the higher dose (50 μM) two sites had mutation hotpots. These were at positions 156 and 168, both of which were at GC basepairs. When all treatments were combined an extra hotspot at position 155 is seen (also a GC basepair). There are no sites for hotspots which correspond to those seen in the single and tandem substitution mutation spectra (Figure 4.9).

Non-treated Control



10 μM α-Acetoxytamoxifen



25 μM α-Acetoxytamoxifen

50 μM α-Acetoxytamoxifen

α-Acetoxytamoxifen combined

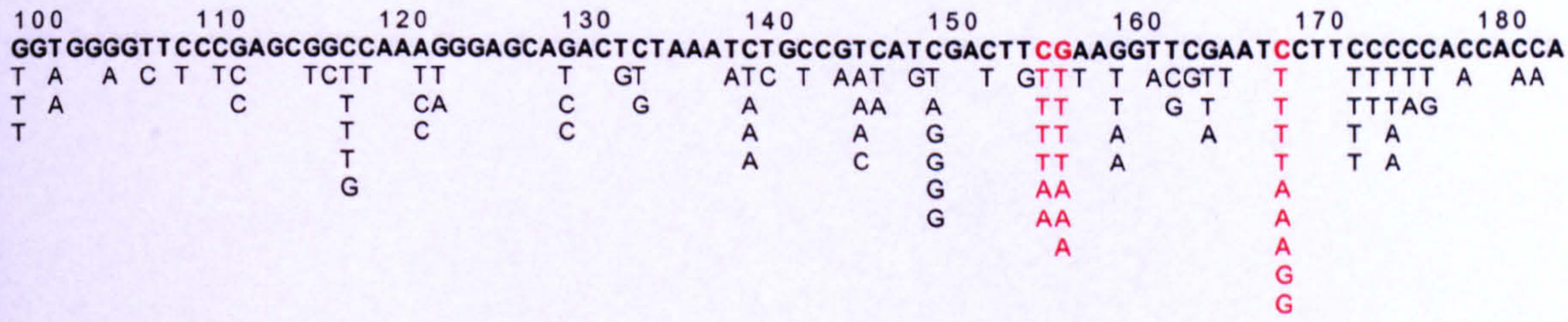


Figure 4.10 Mutation spectra depicting multiple substitutions induced after treatment with α-acetoxytamoxifen or in ethanol treated control (10 μM, 25 μM and 50 μM). Hotspots are shown in red.

4.3.3 Part B: Mutagenicity of plasmid treated with 4-OHtamQM and replicated in Ad293 cells

4.3.3.1 Mutation frequency in *supF* gene

Treatment of pSP189 plasmid with 4-OHtamQM induced an increase in mutation frequency for all doses in comparison to solvent control (1:1, ethanol and acetonitrile), as illustrated in Table 4.5. The lower dose of 50 μM increased mutation frequency by 1.9-fold relative to the solvent control. The intermediate dose of 100 μM increased mutation frequency by 19.5-fold over that of the solvent control, whilst the highest dose (250 μM) increased mutation frequency by 21.5-fold compared to the solvent control. Interestingly the mutation frequency of plasmid treated with water only was 0.2×10^{-4}, 36.5-fold lower than solvent treated control.

Table 4.5. Mutation frequency induced by 4-hydroxytamoxifen quinone methide replicated in Ad293 cells

Treatment	Colonies Screened	Number of Mutants	Mutation Frequency[a]	Adduct Number[b] (± S.D.)
Solvent Control[c]	54000	18	3.3	0 ± 0
50 μM	61000	38	6.2	5 ± 0.6
100 μM	5400	35	64.4	8 ± 2.3
250 μM	11200	81	72.1	20 ± 1

[a] Mutation frequency per 10^4 colonies

[b] Adduct number per 10^6 nucleotides

[c] Mutation frequency of plasmid dissolved in water only was 0.2×10^{-4}

4.3.3.2 Mutation types found in *supF* gene

Table 4.6 shows the types of sequence alterations in the *supF* gene of pSP189 plasmid treated with 4-OHtamQM. It can be seen that for all doses, including solvent control,

base substitutions are the major type of mutation seen accounting for between 53% - 74% of all plasmids sequenced. At all treatment levels, including solvent control, either single or multiple base substitution mutations were most prevalent. Tandem substitutions only appeared in a single mutant plasmid at the highest dose (250 μM). Of the frameshift mutations, insertions were relatively uncommon, only accounting for up to 3.7% of all mutations in the treated plasmid, and 11% in the solvent control. Deletions were much more prevalent, especially in the form of large (greater than two adjacent bases deleted) deletions. The proportion of large deletions ranged from 21% at 50 μM to 40% at 250μM, with solvent control plasmid having 28%.

Table 4.6. Types of sequence alterations in *supF* gene of pSP189 plasmids treated with 4-hydroxytamoxifen quinone methide replicated in Ad293 cells

Mutations	Number of plasmids with mutations (%)			
	Control	50 μM	100 μM	250 μM
Base substitutions	**10** (56)	**28** (74)	**25** (71)	**43** (53)
Single	5 (28)	18 (47)	12 (34)	19 (23)
Tandem	0 (0)	0 (0)	0 (0)	1 (1.2)
Multiple	5 (28)	10 (26)	13 (37)	23 (28)
Frameshifts	**8** (44)	**10** (26)	**10** (29)	**38** (47)
Single base deletion	1 (5.6)	1 (2.6)	0 (0)	2 (2.5)
>2 bases deletion	5 (28)	8 (21)	9 (26)	32 (40)
Single base insertion	0 (0)	1 (2.6)	1 (2.9)	1 (1.2)
>2 bases insertion	2 (11)	0 (0)	0 (0)	3 (3.7)
Total plasmids sequenced	**18** (100)	**38** (100)	**35** (100)	**81** (100)

Table 4.7 illustrates the types of single base substitution mutations induced by the different treatments with 4-OHtamQM. There were only a small number of single base substitutions seen in the mutant plasmids sequenced for the control sample. Transversions are the major type of base substitution mutation in solvent control, 50 μM and 250 μM treated plasmid samples. In the 100 μM treated plasmid samples, there were equal proportions of both transversions and transitions. Of the transversions, for the solvent control plasmid sample all four possibilities are seen

(one mutation each for GC→TA, GC→CG, AT→TA and AT→CG) along with a single GC→AT transition.

Table 4.7. Types of single base substitution mutations in *supF* gene of pSP189 plasmids treated with 4-hydroxytamoxifen quinone methide replicated in Ad293 cells

Mutations	Number of plasmids with mutations (%)			
	Control	50 μM	100 μM	250 μM*
Transversions	**4** (80)	**11** (61)	**6** (50)	**12** (57)
GC→TA	1 (20)	4 (22)	3 (25)	7 (33)
GC→CG	1 (20)	6 (33)	3 (25)	4 (19)
AT→TA	1 (20)	0 (0)	0 (0)	1 (4.8)
AT→CG	1 (20)	1 (5.6)	0 (0)	0 (0.0)
Transitions	**1** (20)	**7** (39)	**6** (50)	**9** (43)
GC→AT	1 (20)	6 (33)	6 (50)	8 (38)
AT→GC	0 (0)	1 (5.6)	0 (0)	1 (5)
Total single base substitutions	**5** (100)	**18** (100)	**12** (100)	**21** (100)

* includes one tandem substitution

When the plasmid was treated with 4-OHtamQM, there was a preference for transversions at GC base pairs, with GC→TA transversions accounting for between 22% and 33%, and GC→CG transversions for between 19% and 33%. Of the transition mutations occurring, GC→AT was the most prevalent, seen in between 33 and 50% of all single base substitution mutations, with AT→GC only appearing in two plasmids at 50 μM and 250 μM treatments.

A graphical representation of the different single base substitution mutation types induced by 4-OHtamQM in comparison to control is illustrated in Figure 4.11. As the dose of 4-OHtamQM increases so does the proportion of GC→TA transversions. At the same time the proportion of GC→CG transversions decreases. There is a small level of transversions at AT basepairs which doesn't significantly change as the dose increases. The proportion of transitions, both at GC basepair (the majority) and AT basepairs does not vary with the concentration of 4-OHtamQM administered.

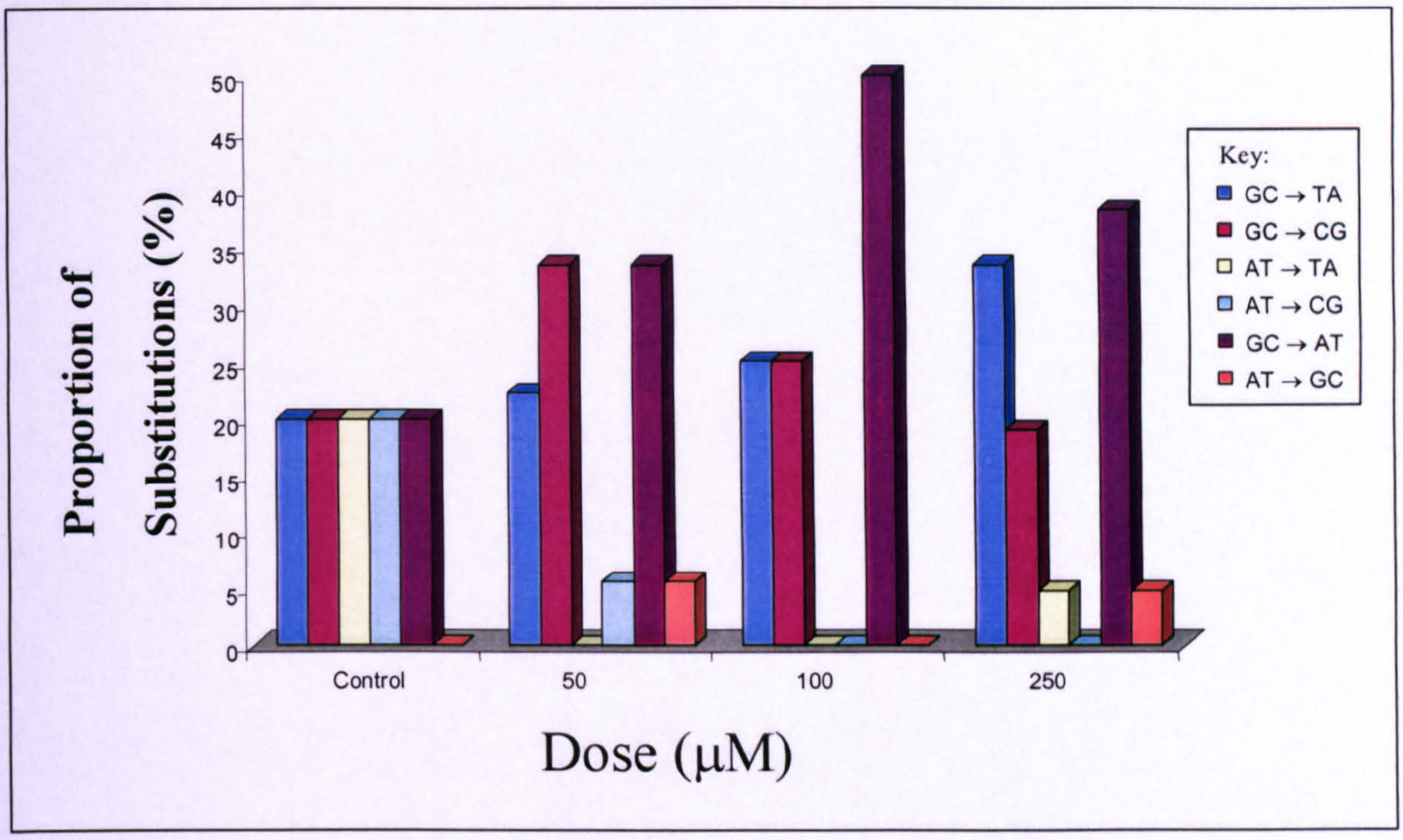


Figure 4.11 Bar chart illustrating the proportion of the different single base and tandem substitution mutations seen in solvent treated control mutants and in those induced by treatment with 4-OHtamQM (50 μM, 100 μM and 250 μM).

Table 4.8 illustrates the types of substitution mutations induced in plasmids with multiple mutations and shows that there is a slightly higher preference for transversions, in comparison to the single base substitutions seen in Table 4.7. Of these transversions there is an increased preference for GC→TA mutations (between 38% and 42% for all treatments, including control). The AT→TA transversion is seen comparatively frequently (31% in control plasmid and between 11% and 26% in treated plasmid). The proportion of transition mutations has decreased in comparison to the single base substitutions seen in Table 4.7. Between 33% and 38% of treated multiple mutations were transitions (31% in control). Of these transitions the majority were GC→AT (between 27% and 35% for treated plasmid, 25% for control) although

in this case the less popular AT→GC transition was seen in all plasmid treatments (2.3% to 7%) including control (6.3%).

Table 4.8. Types of multiple base substitution mutations in *supF* gene of pSP189 plasmids treated with 4-hydroxytamoxifen quinone methide replicated in Ad293 cells

Mutations	Number of plasmids with mutations (%)			
	Control	50 μM	100 μM	250 μM
Transversions	**11** (69)	**29** (67)	**36** (65)	**56** (62)
GC→TA	6 (38)	18 (42)	22 (40)	35 (38)
GC→CG	0 (0)	0 (0)	6 (11)	9 (10)
AT→TA	5 (31)	11 (26)	8 (15)	10 (11)
AT→CG	0 (0)	0 (0)	0 (0)	2 (2.2)
Transitions	**5** (31)	**14** (33)	**19** (35)	**35** (38)
GC→AT	4 (25)	13 (30)	15 (27)	32 (35)
AT→GC	1 (6.3)	1 (2.3)	4 (7.3)	3 (3)
Total multiple base substitutions	**16** (100)	**43** (100)	**55** (100)	**91** (100)

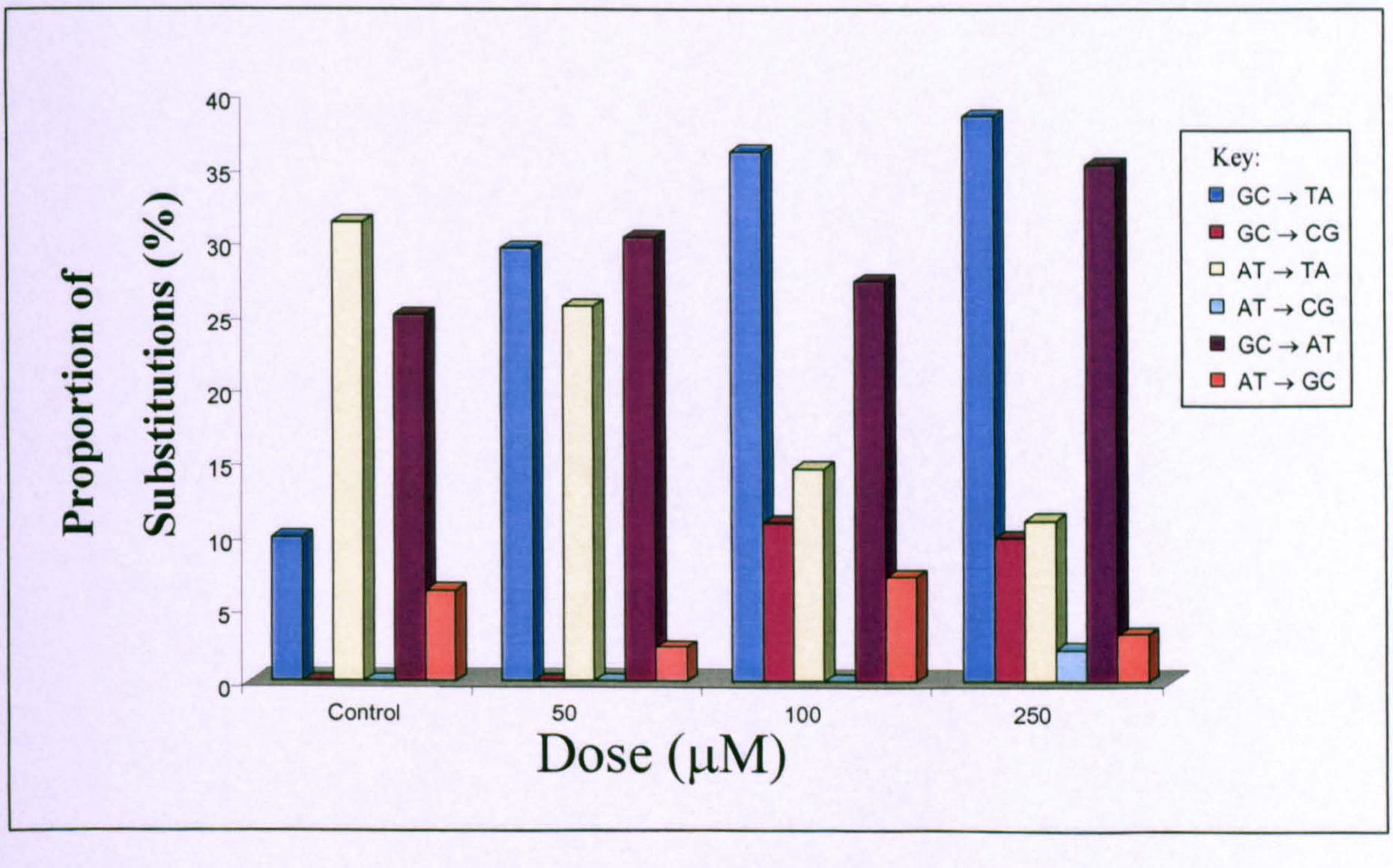
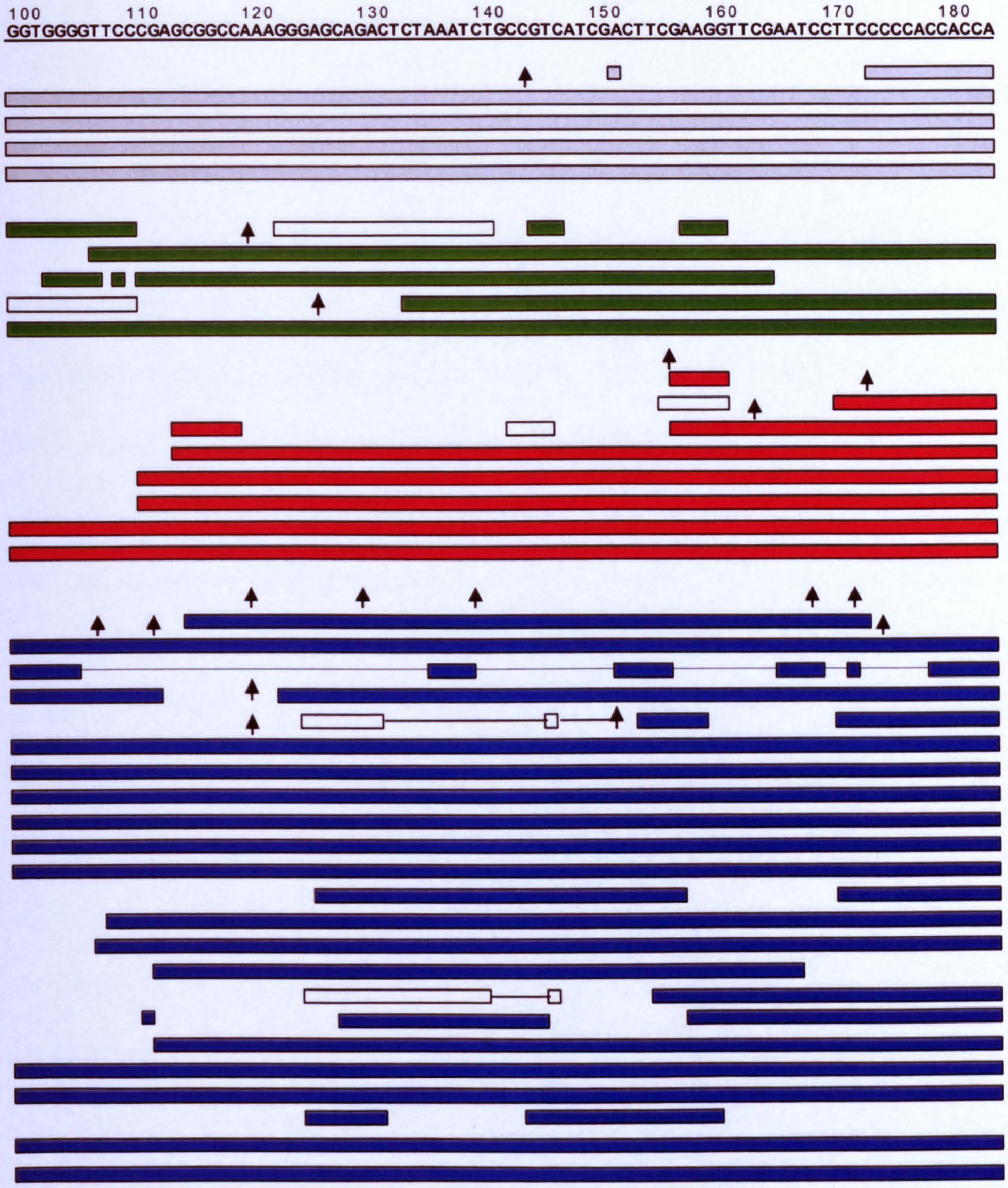


Figure 4.12 Bar chart illustrating the proportion of the different multiple base substitution mutations seen in solvent treated control mutants and in those induced by treatment with 4-OHtamQM (50 μM, 100 μM and 250 μM).

The proportion of multiple substitutions induced by 4-OHtamQM is illustrated graphically in Figure 4.12. Definite trends can be seen in relation to AT→TA and GC→TA transversions and to a lesser extent GC→AT transitions. As the dose of 4-OHtamQM increases over control the proportion of AT→TA transversions decreases and the proportion of GC→TA transversions increases. The proportion of GC→AT transitions increases with dose of 4-OHtamQM, but in a non linear way.

Of the frameshift mutations detected, insertions were relatively uncommon, only accounting for up to 3.7% of all mutations in the treated plasmid, and 1 1% in the control (Table 4.6). Deletions were much more prevalent, especially in the form of large (greater than two adjacent bases deleted) deletions. The proportion of large deletions ranged from 21% at 50 μM to 40% at 250μM, with control plasmid having 28%. Figure 4.13 illustrates the different insertions and deletions, including their sequence and position in the *supF* gene.

Figure 4.13 (overleaf) Illustrates the deletions seen in solvent control mutants and in those induced by treatment with 4-OHtamQM (50 μM, 100 μM and 250 μM).



Key: Deletions: Control 50 μM 100 μM 250 μM

Denotes a deletion which is part of a multiple mutation.

Lines joining deletions show that both mutations appeared in the same plasmid.

Denotes insertion: Control; AATGC @ 143, and 1 large insertion covering *supF*.

50 μM; A @ 119, AGC @ 125, as part of a multiple mutation.

100 μM; C @ 172,T @ 156 as part of multiple mutation,TCGAAGGTT @ 162 as part of multiple mutation.

250 μM; T @ 129, AAGCC @ 119 (x 2), CCCTT @ 171.

These were part of multiple mutations: TT @ 173, A @ 168, TTCCT @ 111, A @ 149, AGC @ 119, G @ 116 and C @ 139 in same plasmid.

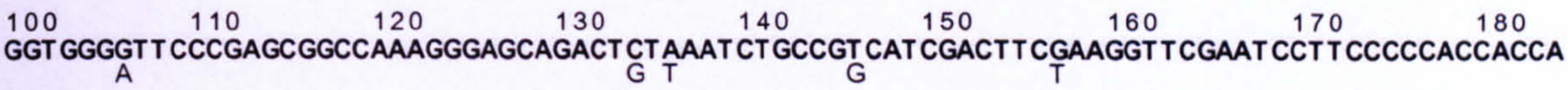
Figure 4.13.

4.3.3.3 Mutation spectra in *supF* gene

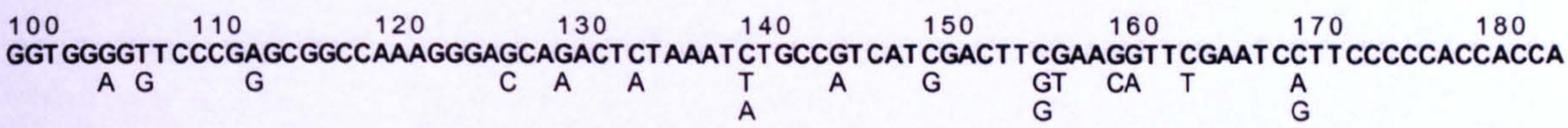
The distribution of single base and tandem substitution mutations within the *supF* gene for plasmids dosed with 4-OHtamQM (50 μM, 100 μM and 250 μM) or control treatments is shown in Figure 4.14. Treated spectra are found to be significantly different from control (p (same) ≤ 0.05). In the mutation spectra induced by control treatment with solvent only or at the lower dose (50 μM), there are no hotspots. At the intermediate dose (100 μM) there is a single hotspot at position 129. At the highest dose (250 μM) there are two hotspots; one at position 129, again, and another new one at 156. If all three treatments are combined there are now four hotspots at positions 129, 139, 155 and 156. All mutation hotspots are at positions of GC basepairs.

The distribution of multiple substitution mutations within the *supF* gene for plasmids dosed with 4-OHtamQM (50 μM, 100 μM and 250 μM) or control treatments is shown in Figure 4.15. There is a single mutation hotspot in the control (solvent only) treated plasmid at position 156. This is also present in the multiple mutation spectrum induced by the 50 μM dose, along with additional hotspots at positions 133, 135 and 174. The 100 μM dose induces hotspots at positions 100, 133, 156, 173 and 174, whilst the highest dose (250 μM) induces mutation hotspots at positions 100, 133, 156, and 174. Positions 100, 133, 156, and 174 appear in more than one of the mutation spectra. The hotspot at position 156 is actually seen in all multiple mutation spectra, including control. All hotspots occur at GC basepairs, apart from the hotspot at the AT basepair at position 135 in the plasmid treated with 50 μM 4-OHtamQM.

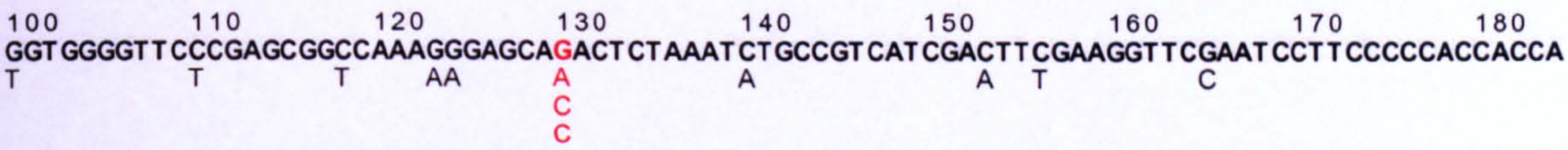
Non-treated Control



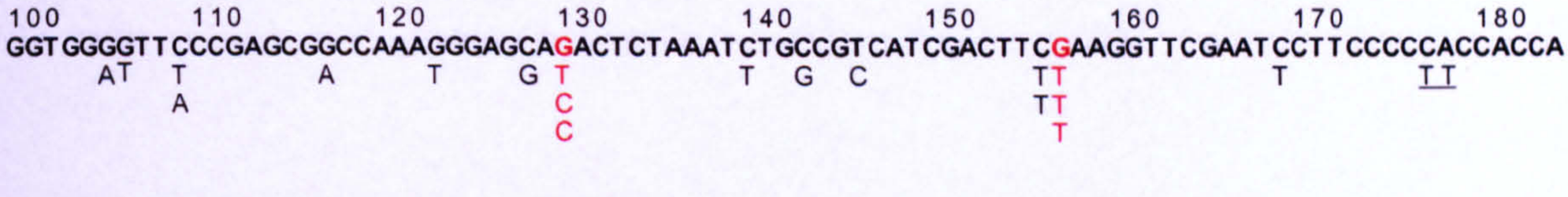
50 μM 4-Hydroxytamoxifen Quinone Methide



100 μM 4-Hydroxytamoxifen Quinone Methide



250 μM 4-Hydroxytamoxifen Quinone Methide



4-Hydroxytamoxifen Quinone Methide Combined

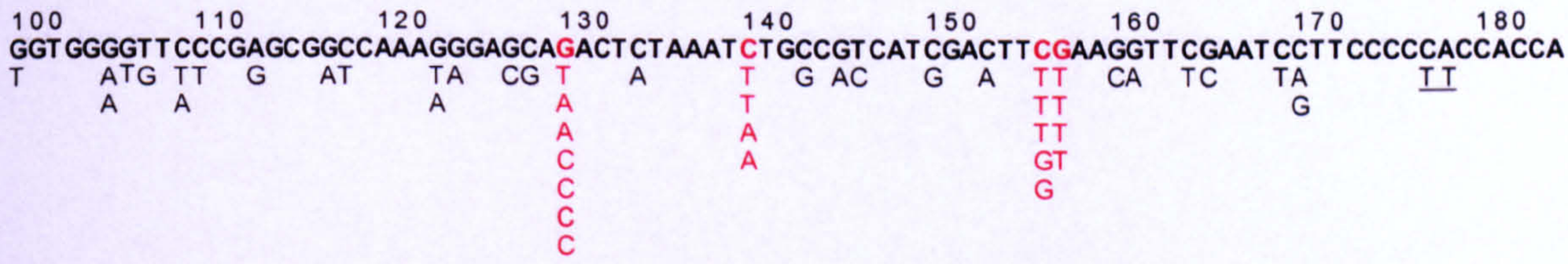


Figure 4.14 Mutation spectra depicting single base and tandem substitutions induced in solvent control or after treatment with 4-OHtamQM (50 μM, 100 μM and 250 μM). Hotspots are shown in red.

Non-treated Control

50 μM 4-Hydroxytamoxifen Quinone Methide

100 μM 4-Hydroxytamoxifen Quinone Methide

250 μM 4-Hydroxytamoxifen Quinone Methide

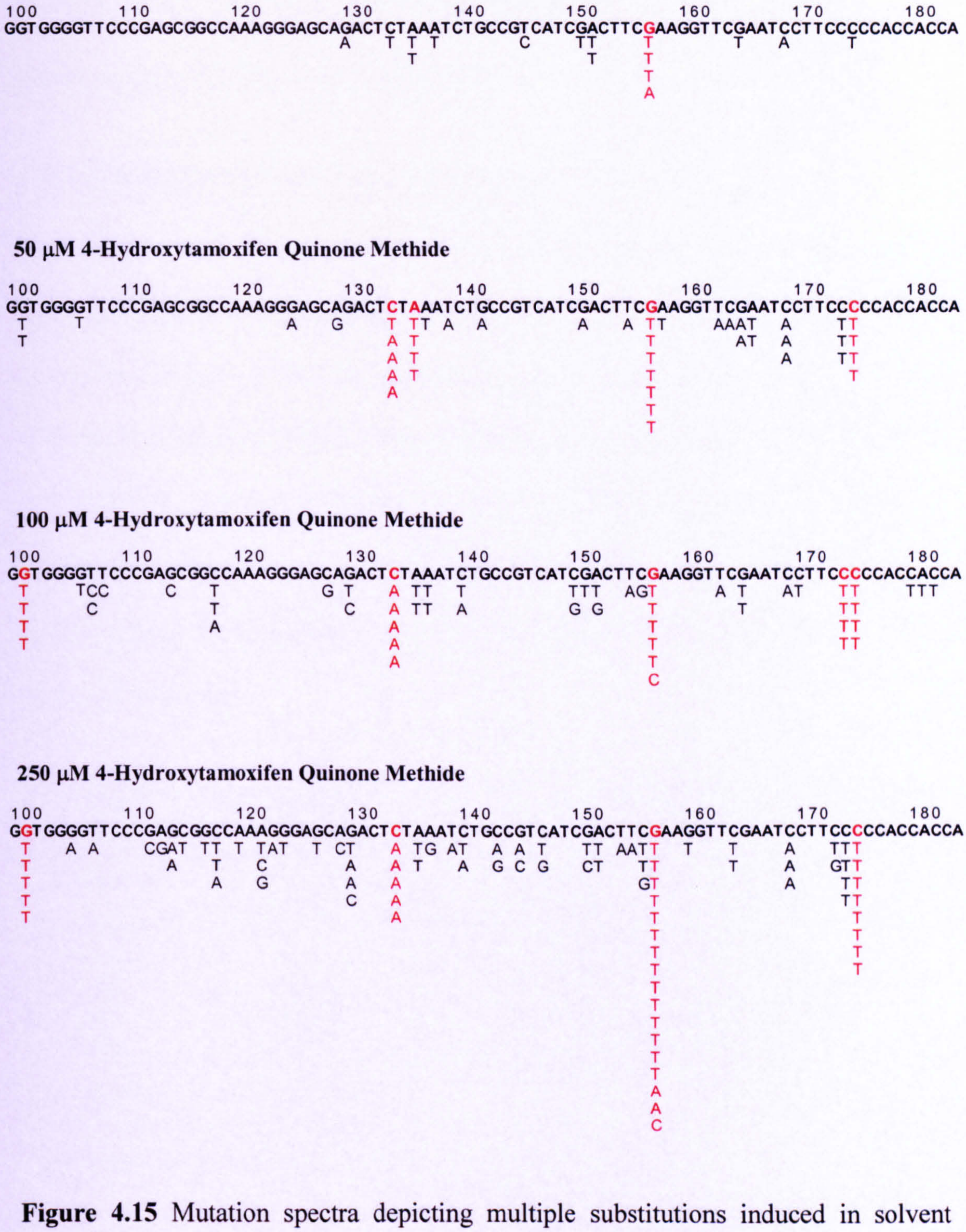


Figure 4.15 Mutation spectra depicting multiple substitutions induced in solvent control or after treatment with 4-OHtamQM (50 μM, 100 μM and 250 μM). Hotspots are shown in red.

4.3.4 Part C: Mutagenicity of plasmid treated with α-acetoxytamoxifen and replicated in GM00637 normal cells

4.3.4.1 Mutation frequency in *supF* gene

Treatment of pSP189 plasmid with α-acetoxytamoxifen induced an increase in mutation frequency for all doses in comparison to solvent control (ethanol only), as illustrated in Table 4.9. The mutation frequency for control plasmid was much larger than the spontaneous mutation frequency of plasmid in water (0.4×10^{-4}). The lower dose of 10 μM increased mutation frequency by 1.26-fold relative to the control. The intermediate dose of 25 μM increased mutation frequency by 1.27-fold over that of control, whilst the highest dose (50 μM) increased mutation frequency by 1.7-fold compared to control.

Table 4.9. Mutation frequency induced by α-acetoxytamoxifen replicated in GM00637 cells

Treatment	Colonies Screened	Number of Mutants	Mutation Frequency[a]	Adduct Number[b] (± S.D.)
Solvent Control[c]	2600	3	11.5	0 ± 0
10 μM	38000	55	14.5	50 ± 8.8
25 μM	22000	31	14.7	160 ± 8
50 μM	27000	51	19	240 ± 52

[a] Mutation frequency per 10^4 colonies

[b] Adduct number per 10^6 nucleotides

[c] Mutation frequency of plasmid dissolved in water only was 0.4×10^{-4}

4.3.4.2 Mutation types found in *supF* gene

Due to the low spontaneous mutation frequency induced by ethanol treated control plasmid (only 3 mutants were found after sequencing); a mutation spectrum has been

compiled from previously published *supF* studies using GM00637 cells (Seidman, *et al.*, 1987 and Myrand, *et al.* 1996). All spontaneous mutations, (plasmid in TE buffer) are taken from the mutation spectrum compiled from these two reports.

Table 4.10. Types of sequence alterations in *supF* gene of pSP189 plasmids treated with α-acetoxytamoxifen replicated in GM00637 cells

Mutations	Number of plasmids with mutations (%)			
	Control	10 μM	25 μM	50 μM
Base substitutions	**61** (95)	**53** (96)	**28** (90)	**48** (94)
Single	51 (80)	42 (76)	18 (58)	41 (80)
Tandem	1 (9.1)	1 (1.8)	1 (1.5)	0 (0)
Multiple	9 (14)	10 (18)	9 (29)	7 (14)
Frameshifts	**3** (4.7)	**2** (3.6)	**3** (9.7)	**3** (5.9)
Single base deletion	3 (4.7)	0 (0)	1 (3.2)	0 (0)
>2 bases deletion	0 (0)	2 (3.6)	1 (3.2)	2 (3.9)
Single base insertion	0 (0)	0 (0)	1 (3.2)	1 (2)
>2 bases insertion	0 (0)	0 (0)	0 (0)	0 (0)
Total plasmids sequenced	**64** (100)	**55** (100)	**31** (100)	**51** (100)

For all doses of α-acetoxytamoxifen and the control base substitutions are the major type of mutation seen accounting for between 90% and 96% of all plasmids sequenced (Table 4.10). Single base substitutions were most prevalent, between 58% and 80% in treated plasmid, and 80% in control plasmid. Tandem substitutions only appeared in single mutant plasmids for control and the two lower treatments (10 μM and 25 μM). Multiple base substitution mutations accounted for 14% of all mutations in control treated plasmid and between 14% and 29% in α-acetoxytamoxifen treated plasmid. Of the frameshift mutations, insertions were relatively uncommon, only appearing in 25 μM and 50 μM treated plasmids (3.2% and 2%, respectively). Deletions were more prevalent. In control plasmid 4.7% of all mutant plasmids had single base deletions, and 3.2% in plasmid treated with 25 μM α-acetoxytamoxifen. Large deletions appeared in all treated plasmids (3.2% to 3.9%) but not in controls.

The single and tandem base substitution mutations can be further categorised into transversions or transitions (Table 4.11).

Table 4.11. Types of single and tandem base substitution mutations in *supF* gene of pSP189 plasmids treated with α-acetoxytamoxifen replicated in GM00637 cells

Mutations	Number of plasmids with mutations (%)			
	Control	10 μM	25 μM	50 μM
Transversions	**25** (47)	**15** (34)	**10** (50)	**26** (63)
GC→TA	14 (26)	9 (20)	3 (15)	17 (41)
GC→CG	9 (17)	6 (14)	7 (35)	7 (17)
AT→TA	1 (2)	0 (0)	0 (0)	2 (4.9)
AT→CG	1 (2)	0 (0)	0 (0)	0 (0.0)
Transitions	**28** (53)	**29** (66)	**10** (50)	**15** (37)
GC→AT	28 (53)	27 (61)	10 (50)	15 (37)
AT→GC	0 (0)	2 (4.5)	0 (0)	0 (0)
Total single base substitutions	**53** (100)	**44** (100)	**20** (100)	**41** (100)

In control plasmid the majority of single base substitutions were in the form of GC→AT transitions (53% of all substitutions). The next most prevalent substitutions being GC→AT transversions (26%) followed by GC→CG transversions (17%). In the treated plasmid, at the two lower doses, there was also a preference for GC→AT transitions (61% for 10 μM and 50% for 25 μM). At these doses, GC→TA transversions accounted for between 15% and 20% and GC→CG transversions for between 14% and 35% of mutations. At the highest dose (50 μM) there was, however, a slight preference for GC→TA transversions in 41% of all substitution mutations. GC→AT transitions accounted for 37% and GC→CG transversions for 17% at this dose level. Transversions and transitions at AT basepairs were uncommon at all doses, accounting for less than 5% in each case. Figure 4.16 illustrates the proportion of the different single base substitutions seen in the form of a bar chart. There are no clear trends to be noted, but it is clear that GC→AT transition mutations are important at all doses, including control.

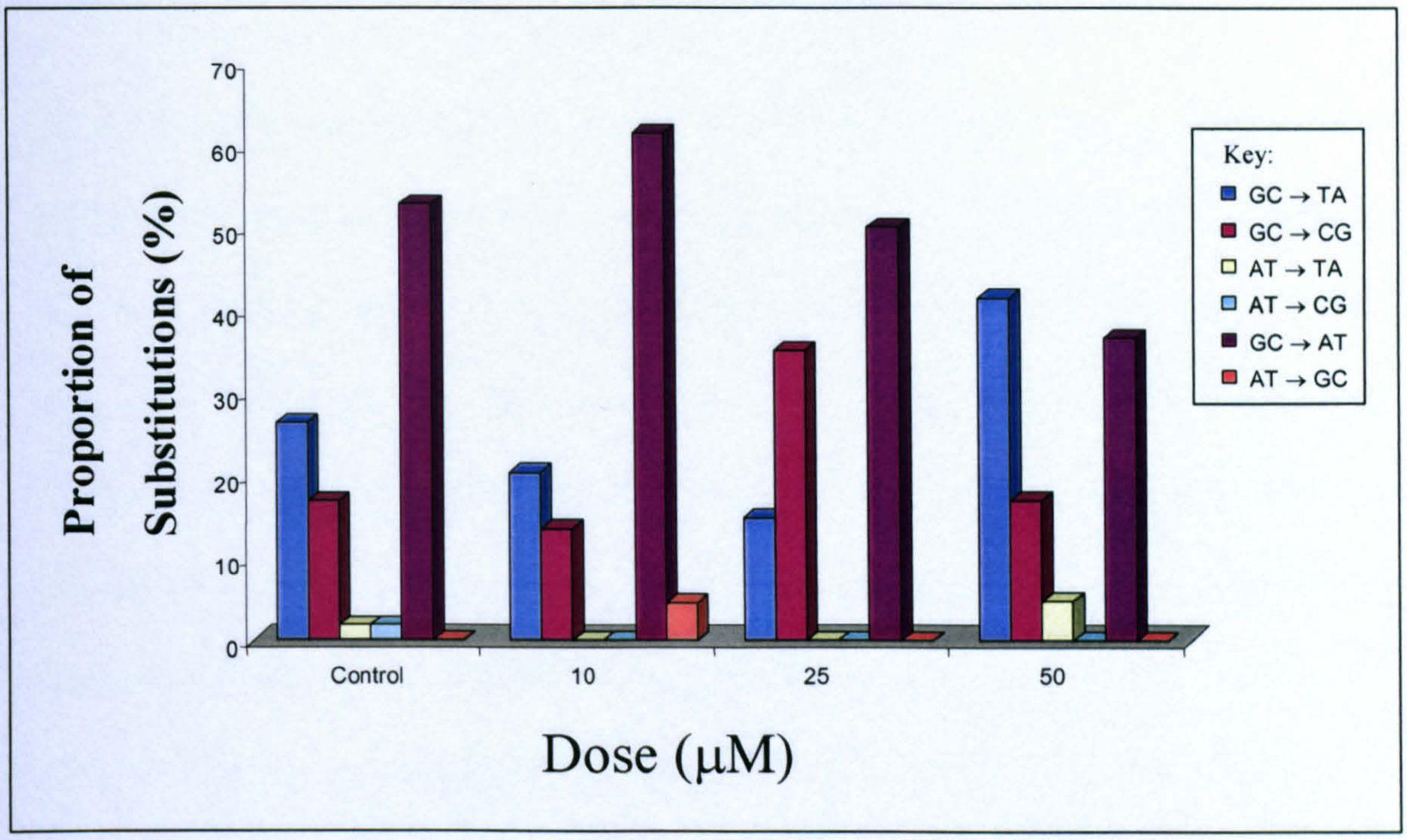


Figure 4.16 Bar chart illustrating the proportion of the different single base and tandem substitution mutations seen in spontaneous mutants and in those induced by treatment with α-acetoxytamoxifen (10 μM, 25 μM and 50 μM) replicated in GM00637 cells.

Table 4.12. Types of multiple base substitution mutations in *supF* gene of pSP189 plasmids treated with α-acetoxytamoxifen replicated in GM00637 cells

Mutations	Number of plasmids with mutations (%)			
	Control	10 μM	25 μM	50 μM
Transversions	**13** (81)	**23** (56)	**21** (58)	**12** (71)
GC→TA	9 (56)	9 (22)	9 (25)	8 (47)
GC→CG	3 (19)	9 (22)	9 (25)	3 (18)
AT→TA	1 (6)	4 (9.8)	3 (8.3)	0 (0.0)
AT→CG	0 (0)	1 (2.4)	0 (0)	1 (5.9)
Transitions	**3** (19)	**18** (44)	**15** (42)	**5** (29)
GC→AT	2 (13)	13 (32)	11 (31)	5 (29)
AT→GC	1 (6.3)	5 (12)	4 (11)	0 (0)
Total multiple base substitutions	**16** (100)	**41** (100)	**36** (100)	**17** (100)

The multiple base substitutions induced in control treated plasmid were mainly transversions (81%) (Table 4.12). GC→TA transversions were the main mutations

seen, followed by GC→CG transversions (19%) and GC→AT transitions (13%). When plasmid was treated with α-acetoxytamoxifen the majority of multiple base substitutions were in the form of transversions (between 56% and 71%). However, the most prevalent substitution at the 10 μM and 25 μM doses was the GC→AT transition, accounting for 32% and 31%, respectively. GC→TA and GC→CG transversions were 22% each, and 25% each at 10 μM and 25 μM, respectively. At the higher dose (50 μM) the major substitution was the GC→TA transversion (47%) followed by the GC→AT transition at 29% and GC→CG transversion at 18%. The substitutions at AT basepairs were also relatively uncommon in plasmids with multiple mutations, 12% being the highest proportion achieved.

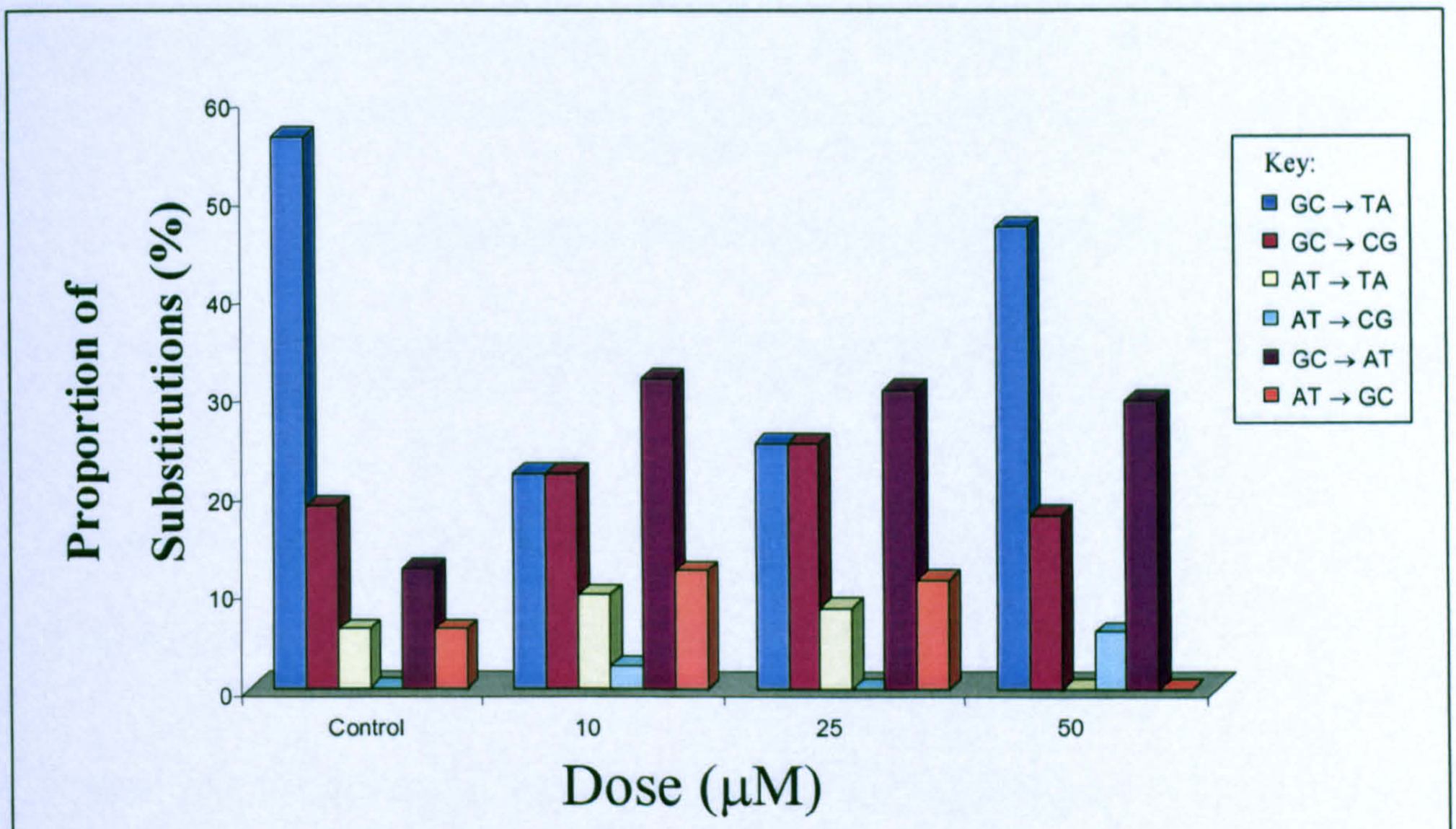


Figure 4.17 Bar chart illustrating the proportion of the different multiple substitution mutations seen in spontaneous mutants and in those induced by treatment with α-acetoxytamoxifen (10 μM, 25 μM and 50 μM) replicated in GM00637 cells.

For multiple substitutions (Figure 4.17) it can be seen that the proportion of GC→TA transversions increases with dose (10 μM to 50 μM α-acetoxytamoxifen). The proportion of GC→AT transitions stays almost constant as dose increases, although it is actually decreasing slightly. There are no trends in relation to the other substitution types.

Frameshift mutations are not induced to any great extent at any of the doses of α-acetoxytamoxifen or in control plasmids. As shown in Table 4.10, the proportion of frameshift mutations is less than 6% for all treatments. The deletions and insertions seen are illustrated in Figure 4.18.

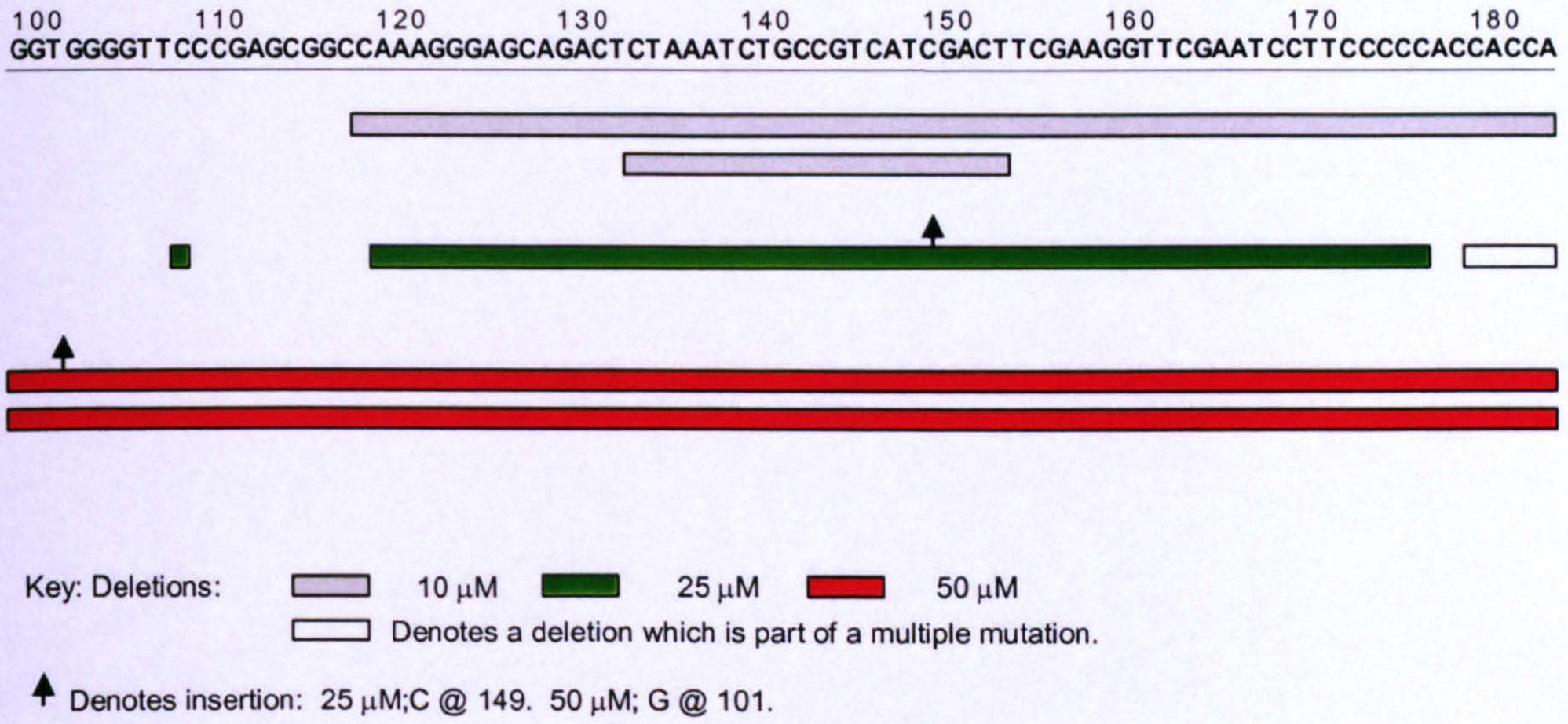


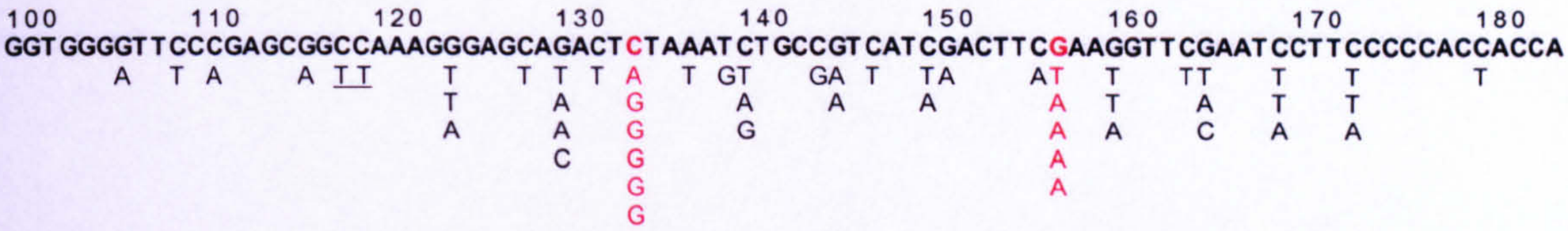
Figure 4.18 Deletions and insertions induced by treatment α-acetoxytamoxifen (10 μM, 25 μM and 50 μM) replicated in GM00637 cells.

4.3.4.3 Mutation spectra in *supF* gene

The distribution of single base and tandem substitution mutations within the *supF* gene for plasmids dosed with α-acetoxytamoxifen (10 μM, 25 μM and 50 μM) or control treatments and replicated in GM00637 cells is shown in Figure 4.19. When these spectra are compared using the hyperg program (Cariello, *et al.*, 1994), treated spectra are found to be significantly different from control (p (same) ≤ 0.05). In the single base substitution mutation spectra induced by spontaneous means there are two hotspots at positions 133 and 156 (Figure 4.19). At the lower dose of α-acetoxytamoxifen (10 μM) there are three mutation hotspots at positions 104, 139, and 168. The dose of 25 μM induces a single mutation hotspot at position 108, and the highest treatment (50 μM) induces a hotspot at position 144. If all single and tandem substitutions from all treated plasmids are combined there are six hotspots at positions 104, 108, 133, 139, 144, and 168. All hotspots whether, or not, induced by treatment with α-acetoxytamoxifen are at positions of GC basepairs.

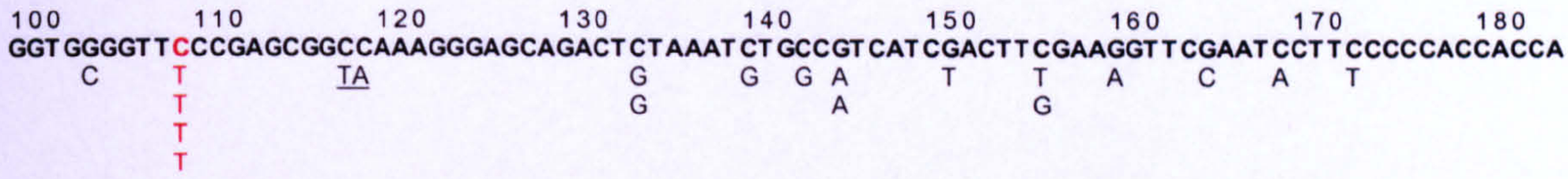
The distribution of multiple substitution mutations within the *supF* gene for plasmids dosed with α-acetoxytamoxifen (10 μM, 25 μM and 50 μM) or control treatments is shown in Figure 4.20. There are two mutation hotspots in the spontaneous spectrum at positions 123 and 129. There are two hotspots at the lowest dose of α-acetoxytamoxifen (10 μM) at positions 133 and 164, whilst the 25 μM dose induces a single hotspot at position 108. There are no hotspots in the mutation spectrum from the highest α-acetoxytamoxifen dose (50 μM). When all treatments are combined this gives six hotspots at positions 108, 129, 133, 149, 156, and 164. All hotspots are at GC basepair sites.

Non-treated Control

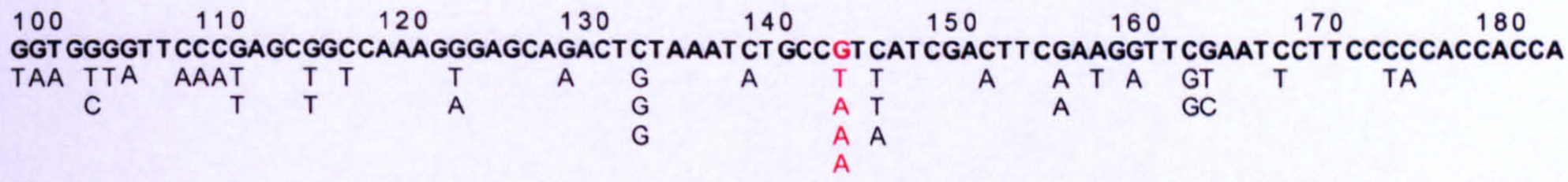


10 μM α-Acetoxytamoxifen

25 μM α-Acetoxytamoxifen



50 μM α-Acetoxytamoxifen



α-Acetoxytamoxifen combined

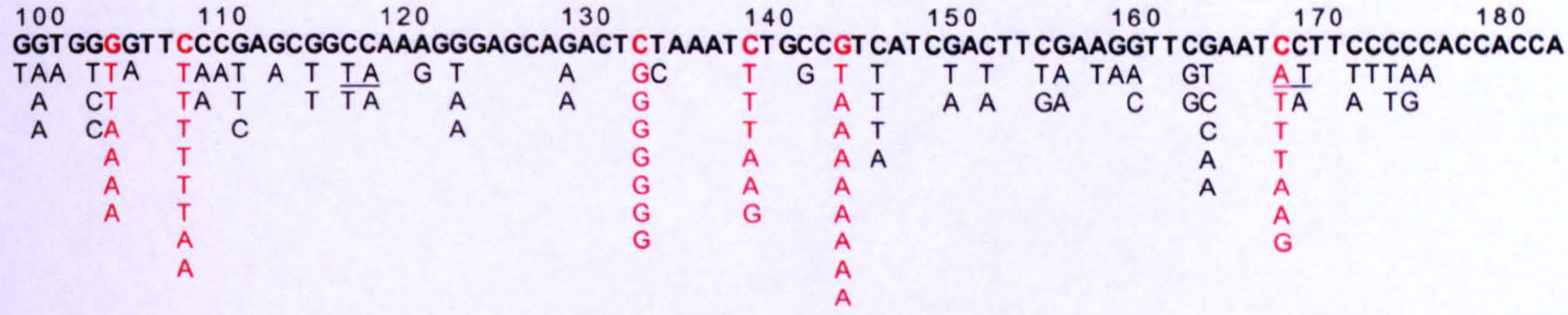
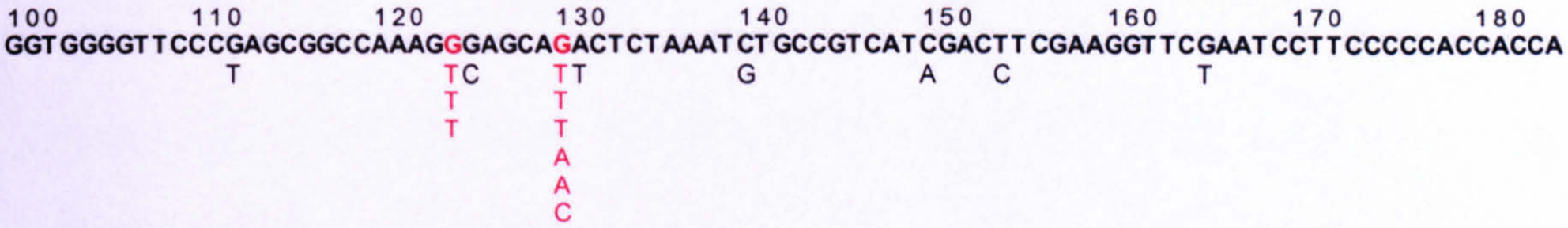
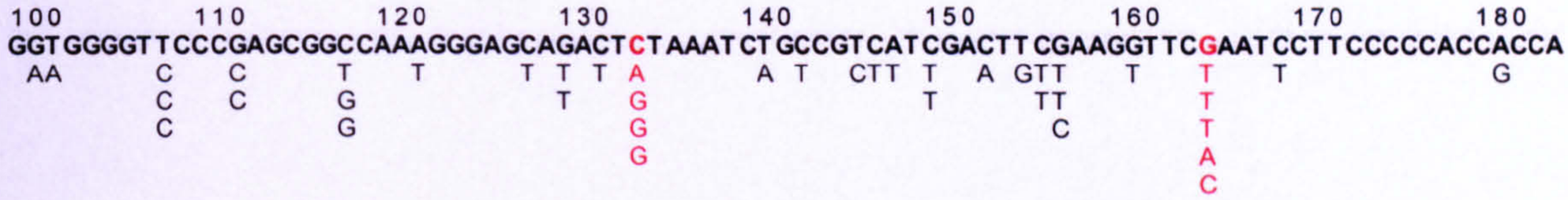


Figure 4.19. Mutation spectra depicting single base and tandem substitutions induced spontaneously and after treatment with α-acetoxytamoxifen (10 μM, 25 μM and 50 μM) replicated in GM00637 cells. Spontaneous spectrum taken from Seidman, *et al.*, 1987 and Myrand, *et al.*, 1996. Hotspots are shown in red.

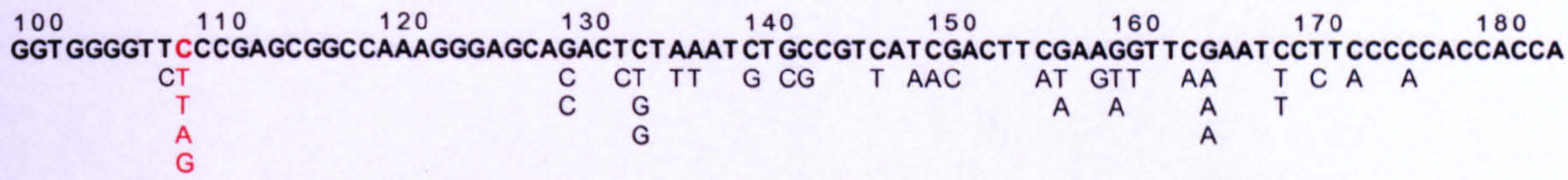
Non-treated Control



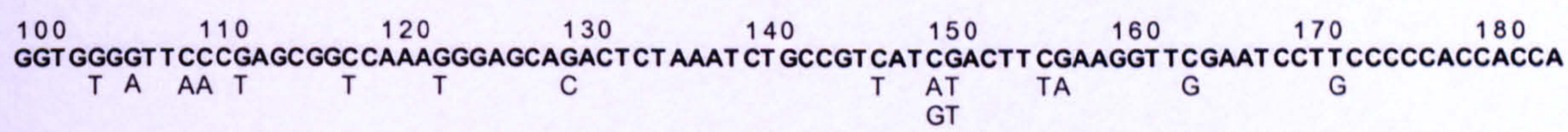
10 μM α-Acetoxytamoxifen



25 μM α-Acetoxytamoxifen



50 μM α-Acetoxytamoxifen



α-Acetoxytamoxifen combined

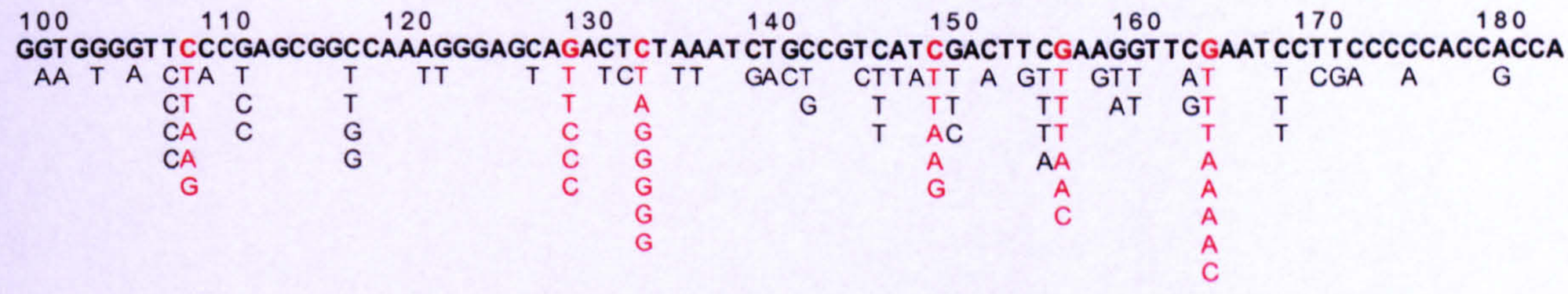


Figure 4.20 Mutation spectra depicting multiple substitutions induced spontaneously and after treatment with α-acetoxytamoxifen (10 μM, 25 μM and 50 μM) replicated in GM00637 cells. Spontaneous spectrum taken from Seidman, *et al.*, 1987. Hotspots are shown in red.

Comparison of the sites of mutation hotspots in GM00637 cells and Ad293 cells, there are no common sites from the single and tandem base substitution mutation spectra (Figures 4.9 and 4.19). In the multiple mutation spectra (Figures 4.10 and 4.20) there is one common hotspot at position 156.

4.3.4 Part D: Mutagenicity of plasmid treated with α-acetoxytamoxifen replicated in GM04429 XPA cells

4.3.4.1 Mutation frequency in *supF* gene

Treatment of pSP189 plasmid with α-acetoxytamoxifen induced an increase in mutation frequency for all doses in comparison to solvent control (ethanol only), as illustrated in Table 4.13.

Table 4.13. Mutation frequency induced by α-acetoxytamoxifen replicated in GM04429 cells

Treatment	Colonies Screened	Number of Mutants	Mutation Frequency[a]	Adduct Number[b] (± S.D.)
Solvent Control[c]	47000	40	8.5	0 ± 0
10 μM	35000	38	10.7	50 ± 8.8
25 μM	6500	9	13.9	160 ± 8
50 μM	44000	63	14.4	240 ± 52

[a] Mutation frequency per 10^4 colonies

[b] Adduct number per 10^6 nucleotides

[c] Mutation frequency of plasmid dissolved in water only was 1.5×10^{-4}

The mutation frequency for ethanol treated control plasmid was much larger than the spontaneous mutation frequency of plasmid in water (1.5×10^{-4}). The lower dose of 10 μM increased the mutation frequency by 1.26-fold compared to the control. The intermediate dose of 25 μM increased mutation frequency by 1.64-fold over that of control, whilst the highest dose (50 μM) increased mutation frequency by 1.7-fold compared to control.

4.3.4.2 Mutation types found in *supF* gene

The majority of ethanol treated control mutations were in the form of single base substitutions (40% of all mutations, Table 4.14) or large (greater than 2 bases) deletions (40% also), followed by multiple mutations (15%) along with a small amount of frameshift mutations, in the form of insertions (both single and multiple base, 2.5% each). Treatment with α-acetoxytamoxifen at the lowest dose (10 μM) preferentially induces single base substitutions (55% of all mutations), with deletions also accounting for a large proportion of the mutations (34%). Multiple mutations are also seen in 11% of screened plasmids. Treatment with α-acetoxytamoxifen at the intermediate dose (25 μM), resulted in a low yield of viable plasmid, and consequentially a low number of mutants (9 in all, 4 of which were single base substitutions, 3 were multiple substitutions and the remaining 2 were large deletions). The highest dose of 50 μM induced single base substitutions in 68% of all sequenced plasmids, 1.6% as tandem substitutions, 9.5% as multiple substitutions and 21% as frameshift mutations (4.8% as single base deletions and 16% as large deletions).

Table 4.14. Types of sequence alterations in *supF* gene of pSP189 plasmids treated with α-acetoxytamoxifen replicated in GM04429 cells

Mutations	Number of plasmids with mutations (%)			
	Control	10 μM	25 μM	50 μM
Base substitutions	**22 (55)**	**25 (66)**	**7 (78)**	**50 (79)**
Single	16 (40)	21 (55)	4 (44)	43 (68)
Tandem	0 (0)	0 (0)	0 (1.5)	1 (1.6)
Multiple	6 (15)	4 (11)	3 (33)	6 (9.5)
Frameshifts	**18 (45)**	**13 (34)**	**2 (22)**	**13 (21)**
Single base deletion	0 (0)	2 (5.3)	0 (0)	3 (4.8)
>2 bases deletion	16 (40)	11 (29)	2 (22)	10 (16)
Single base insertion	1 (2.5)	0 (0)	0 (0)	0 (0)
>2 bases insertion	1 (2.5)	0 (0)	0 (0)	0 (0)
Total plasmids sequenced	**40 (100)**	**38 (100)**	**9 (100)**	**63 (100)**

The types of single and tandem base substitutions induced by α-acetoxytamoxifen treated and ethanol treated control plasmid are illustrated in Table 4.15. Spontaneous control substitutions were preferentially in the form of transitions (56% of all single base substitutions) with the GC→AT transition being more prevalent than the AT→GC transition (44% versus 13%, respectively). Transversions accounted for the remaining 44% of substitution mutations with GC→TA and GC→CG being 19% each, and AT→TA being 6%.

Table 4.15. Types of single and tandem base substitution mutations in *supF* gene of pSP189 plasmids treated with α-acetoxytamoxifen replicated in GM04429 cells t

Mutations	Number of plasmids with mutations (%)			
	Control	10 μM	25 μM	50 μM
Transversions	**7 (44)**	**9 (43)**	**3 (75)**	**30 (67)**
GC→TA	3 (19)	5 (24)	2 (50)	23 (51)
GC→CG	3 (19)	2 (9.5)	1 (25)	4 (9)
AT→TA	1 (6)	0 (0)	0 (0)	2 (4.7)
AT→CG	0 (0)	2 (9.5)	0 (0)	1 (2.3)
Transitions	**9 (56)**	**12 (57)**	**1 (25)**	**15 (33)**
GC→AT	7 (44)	11 (52)	1 (25)	11 (24)
AT→GC	2 (13)	1 (4.8)	0 (0)	4 (9)
Total single base substitutions	**16 (100)**	**21 (100)**	**4 (100)**	**45 (100)**

Treatment with 10 μM α-acetoxytamoxifen induces a high proportion of transitions (57% of all single base substitutions) with the GC→AT transition being much more prevalent than the AT→GC transition (52% versus 4.8%, respectively). Transversions account for the remaining 43% of mutations, 24% as GC→TA, and 9.5% each for GC→CG and AT→CG. As the dose of α-acetoxytamoxifen increases, the proportion of transitions decreases. There were only four single base substitutions sequenced for the intermediate dose of 25 μM, these were two GC→TA and one GC→CG transversions and a single GC→AT transition. At the 50 μM dose the majority of single base substitutions were in the form of transversions (51% as

GC→TA, 9% as GC→CG, 4.7% as AT→TA, and 2.3% as AT→CG). The transitions (33% overall) were divided into 24% as GC→AT and 9% as AT→GC.

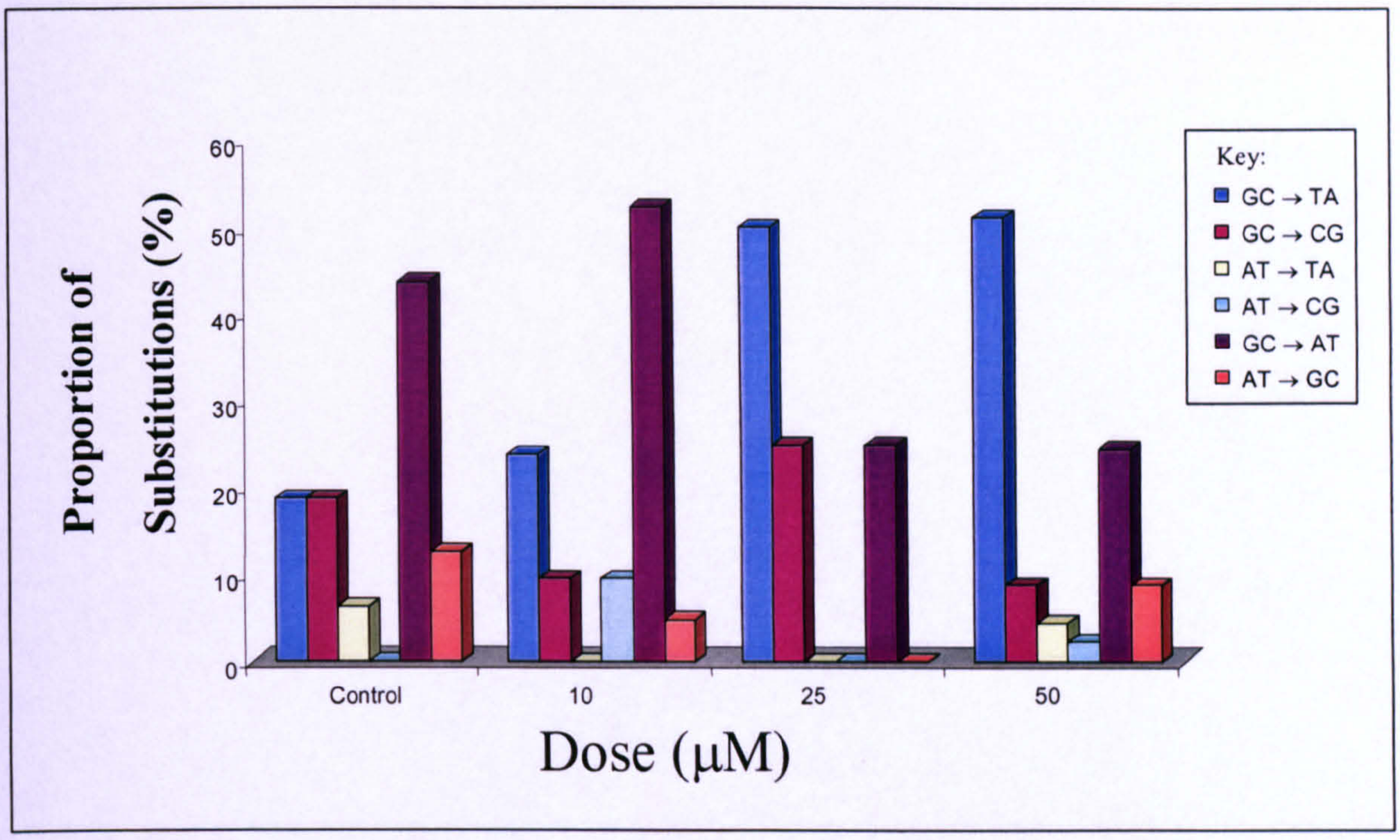


Figure 4.21 Bar chart illustrating the proportion of the different single base and tandem substitution mutations induced in ethanol control or by treatment with α-acetoxytamoxifen (10 μM, 25 μM and 50 μM) replicated in GM04429 cells.

Figure 4.21 illustrates the comparison of the different proportions of base substitution mutations induced in the *supF* gene by the various treatments with ethanol or α-acetoxytamoxifen. It can be seen that the ethanol treated control mutation profile is similar to that of treatment with 10 μM α-acetoxytamoxifen, i.e. a high proportion of GC→AT transitions. The two higher doses of α-acetoxytamoxifen are similar in that they both have an increased proportion of GC→TA transversions.

The types of multiple base substitutions induced by α-acetoxytamoxifen treated and ethanol treated control plasmid are illustrated in Table 4.16.

Table 4.16. Types of multiple base substitution mutations in *supF* gene of pSP189 plasmids treated with α-acetoxytamoxifen replicated in GM04429 cells

Mutations	Number of plasmids with mutations (%)			
	Control	10 μM	25 μM	50 μM
Transversions	**13** (62)	**2** (50)	**0** (0)	**19** (68)
GC→TA	3 (14)	1 (25)	0 (0)	10 (36)
GC→CG	5 (24)	1 (25)	0 (0)	6 (21)
AT→TA	3 (14)	0 (0)	0 (0)	3 (11)
AT→CG	2 (10)	0 (0)	0 (0)	0 (0.0)
Transitions	**8** (38)	**2** (50)	**0** (0)	**9** (32)
GC→AT	4 (19)	0 (0)	0 (0)	6 (21)
AT→GC	4 (19)	2 (50)	0 (0)	3 (11)
Total multiple base substitutions	**21** (100)	**4** (100)	**0** (0)	**28** (100)

There were only significant numbers of multiple mutations found in the control treated plasmid and in plasmid treated with 50 μM α-acetoxytamoxifen. In both cases the predominant type of substitution was the transversion. In control plasmid the GC→CG mutation was most abundant (24% of all multiple mutations seen) followed by GC→TA and AT→TA (14% each) and AT→CG (10%). Both GC→AT and AT→GC transitions accounted for 19% of all multiple mutations seen. In plasmid treated with 50 μM α-acetoxytamoxifen the major mutation was the GC→TA transversion (36%) followed by GC→CG transversion and GC→AT transition (21% each). Both AT→TA (transversion) and AT→GC (transition) mutations accounted for 11% each. At the lower dose of α-acetoxytamoxifen (10 μM) there were only four multiple substitutions seen (one GC→TA transversion, one GC→CG transversion and two AT→GC transitions) and at the 25 μM dose no multiple substitutions were seen.

As can be seen in Figure 4.22 there does not seem to be a trend in respect to the types of multiple mutations seen at the different treatments of plasmid with α-acetoxytamoxifen when replicated inn GM04429 XP cells.

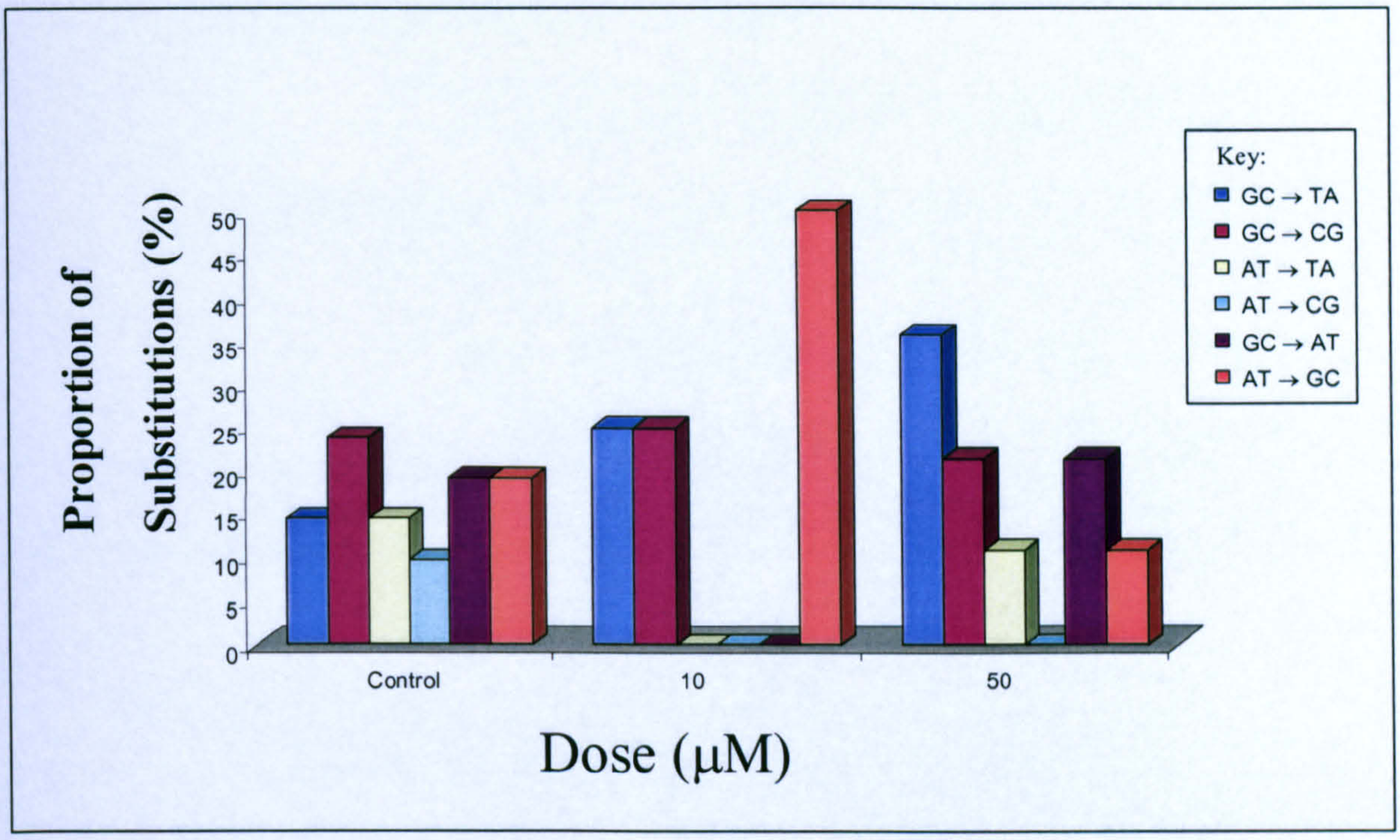


Figure 4.22 Bar chart illustrating the proportion of the different multiple substitution mutations induced in ethanol control or by treatment with α-acetoxytamoxifen (10 μM and 50 μM) replicated in GM04429 cells.

Frameshift mutations account for between 21% and 34% of the treated plasmids sequenced along with 45% of solvent control plasmids sequenced. Referring back to Table 4.13 it can be seen that insertions are only seen in the control samples, and of the deletions it is multiple deletions which predominate. These deletions and insertions seen are illustrated in Figure 4.23.

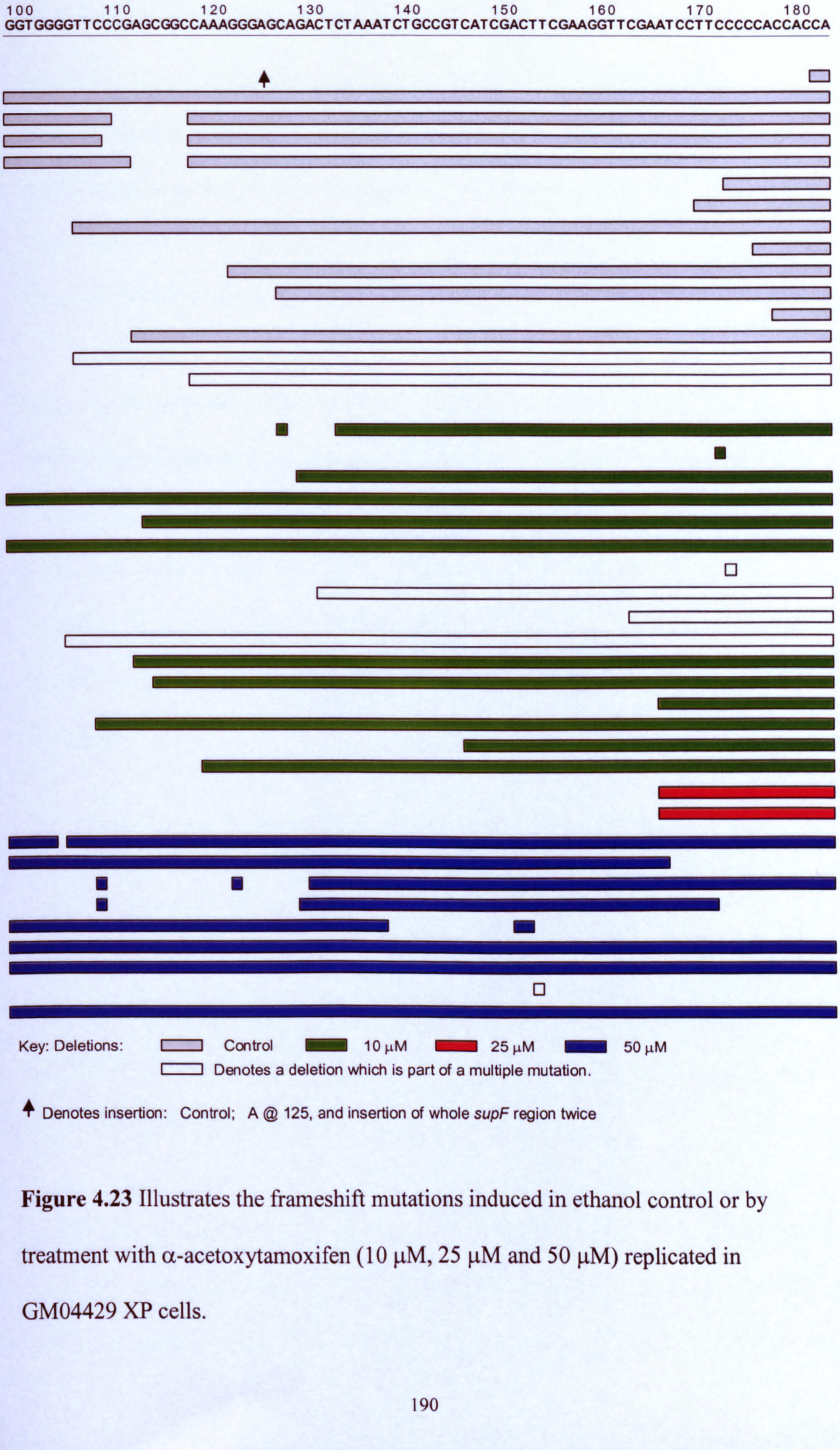


Figure 4.23 Illustrates the frameshift mutations induced in ethanol control or by treatment with α-acetoxytamoxifen (10 μM, 25 μM and 50 μM) replicated in GM04429 XP cells.

4.3.4.3 Mutation spectra in *supF* gene

The distribution of single base and tandem substitution mutations within the *supF* gene for plasmids dosed with α-acetoxytamoxifen (10 μM, 25 μM and 50 μM) or control treatments and replicated in GM04429 (XP) cells is shown in Figure 4.24. When these spectra are compared using the hyperg program treated spectra are found to be significantly different from control (p (same) ≤ 0.05).

In the mutation spectra induced by control treatment with solvent only or at the two lower doses (10 μM and 25 μM) there are no hotspots. At the higher dose (50 μM) there are two hotspots at positions 122 and 175. If all three mutation spectra from α-acetoxytamoxifen treated plasmids are combined there are now four hotspots at positions 122, 129, 164 and 175. Position 122 is a hotspot site in both the 50 μM and combined mutation spectra. All hotspots from treated spectra are at positions of GC base pairs.

Figure 4.25 illustrates the distribution of multiple mutations seen after plasmid treatment with α-acetoxytamoxifen and replication in GM04429 (XP) cells. There were a low amount of multiple mutations induced by this treatment in this cell line, so mutation hotspots only become statistically visible in the combined spectrum (10 μM and 50 μM, as none were seen at 25 μM). These hotspots are at positions 149 and 172, both of which are at GC basepairs

Referring back to the mutation spectra induced by α-acetoxytamoxifen in Ad293 and GM00637 cells it can be observed that there are few similarities in the position of hotspots between the cell lines. In the single and tandem base substitution mutation

spectra (Figures 4.9, 4.19, and 4.24) there is only one common hotspot at position 122, which is seen in Ad293 and GM04429 cells. In the multiple mutation spectra (Figures 4.10, 4.20 and 4.25) there is one common hotspot at position 149 (in GM00637 and GM04429 cells).

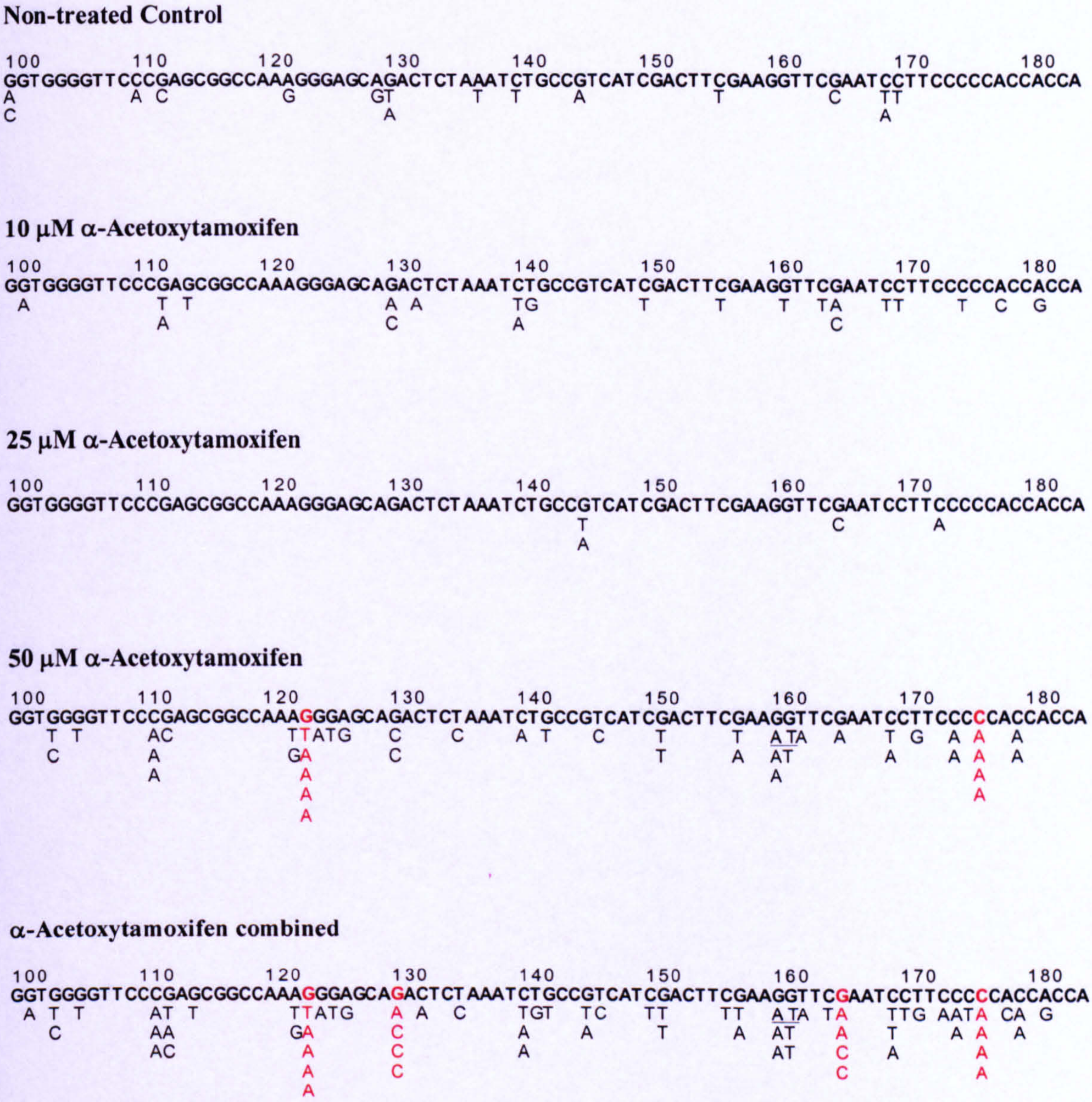
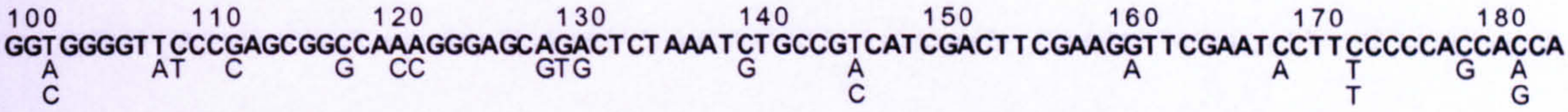
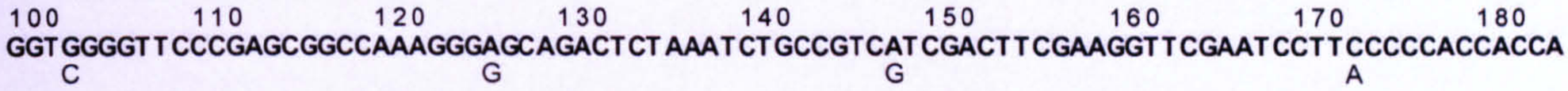


Figure 4.24 Mutation spectra depicting single base and tandem substitution mutations induced in ethanol control or by treatment with α-acetoxytamoxifen (10 μM, 25 μM and 50 μM) replicated in GM04429 cells. Hotspots are shown in red

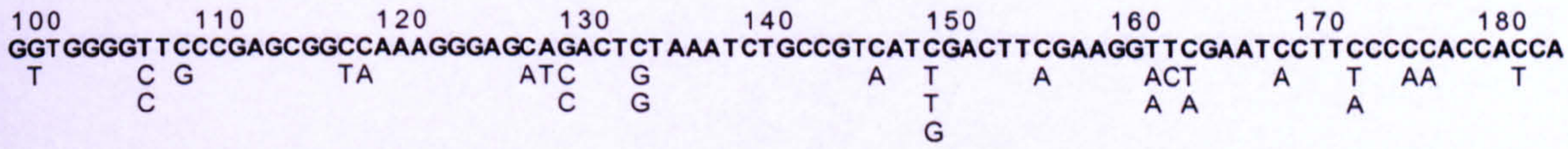
Non-treated Control



10 μM α-Acetoxytamoxifen



50 μM α-Acetoxytamoxifen



α-Acetoxytamoxifen combined

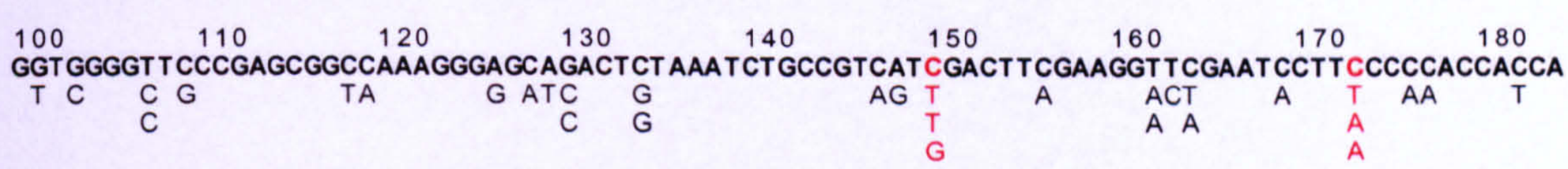


Figure 4.25 Mutation spectra depicting multiple substitution mutations induced in ethanol control or by treatment with α-acetoxytamoxifen (10 μM and 50 μM) replicated in GM04429 cells. Hotspots are shown in red.

4.4 Discussion

The main aim of this chapter was to investigate the types and frequency of mutations induced by DNA adducts formed by two tamoxifen reactive metabolites in human cells. A secondary aim was to determine the role of repair in the mutagenicity of tamoxifen DNA adducts. Several studies have reported on the detection of tamoxifen DNA adducts in rats, mice, and human *in vivo* systems and also in *in vitro* systems (For example; White, *et al.*, 1992; Martin, *et al.*, 1997; Hemminki, *et al.*, 1997; Davies, *et al.*, 1995). To date, however, there are relatively few studies investigating whether the formation of these DNA adducts results in mutations in DNA, particularly for the DNA adducts derived from 4-hydroxytamoxifen. To investigate the relative mutagenicity of tamoxifen DNA adducts α-acetoxytamoxifen and 4-hydroxytamoxifen were used as the DNA damaging agents. α-Acetoxytamoxifen is a model of the reactive metabolite of tamoxifen, α-sulphate tamoxifen (Dasaradhi and Shibutani, 1997), and forms the same α-(N^2-deoxyguanosinyl) tamoxifen DNA adduct as seen in rats, mice and women (Osborne, *et al.*, 1996; Dasaradhi and Shibutani, 1997; Rajaniemi, *et al.*, 1999; Shibutani, *et al.*, 2000a). 4-Hydroxytamoxifen is one of the major metabolites of tamoxifen (Randerath, *et al.*, 1994), which via oxidation to its quinone methide, is believed to form one of the minor tamoxifen DNA adducts (α-(N^2-deoxyguanosinyl) 4-hydroxytamoxifen) in rat liver (Marques and Beland, 1997). In this study the determination of whether the repair of tamoxifen DNA adducts has any effect on their mutagenicity was investigated using repair deficient human fibroblast cells from a patient with *Xeroderma pigmentosum* (GM04429, complementation group A, lacking *XPA* DNA lesion recognition protein) and the equivalent repair proficient cells (GM00637).

Plasmid adducted with α-acetoxytamoxifen was replicated in these two cell lines and the results compared.

4.4.1 Part A and B: Mutagenicity of plasmid treated with α-acetoxytamoxifen or with 4-OHtamQM replicated in Ad293 cells

Replication of adducted plasmid in human Ad293 cells resulted in an increase in mutation frequency above background control levels for both tamoxifen derivatives. Furthermore, mutation frequency increased with higher plasmid adduct levels, which is in contrast to an earlier study from this laboratory carried out in *E-coli* that reported a lack of correlation between total adduct number on the pLIZ lambda shuttle vector and mutagenicity in the *lacI* gene (Lowes, *et al.,* 1999). In the present study, for each compound, at the lower doses used there was only a small increase in mutation frequency above the solvent control. With higher doses, inducing above 160 adducts per 10^6 nucleotides (1.6 adducts per plasmid) in α-acetoxytamoxifen treated plasmid and 8 adducts per 10^6 nucleotides (0.08 adducts per plasmid) in 4-OHtamQM treated plasmids, there is a sharp increase in mutation frequency. This may suggest there is a threshold level of tolerable damage which the cells are able to repair and that at low levels of DNA damage, DNA repair may be induced and the adducts efficiently removed. Higher levels may saturate the available repair mechanisms, resulting in an increase in mutation frequency. The fact that 4-OHtamQM induced such a marked increase in mutation frequency in human cells compared to α-acetoxytamoxifen at the two higher treatment doses suggests that the dG-N^2-4-hydroxytamoxifen DNA adduct is a significantly more mutagenic lesion, which may deserve more consideration than it has so far received in the literature. It has been proposed that the increased mutagenicity of 4-hydroxytamoxifen induced adducts is a consequence of increased

hydrogen bonding potential of dG-N^2-4-OHtamoxifen compared to dG-N^2-tamoxifen due to the presence of the 4-hydroxy group (Lowes, *et al.*, 1999). Extra interactions with complementary bases may disrupt DNA structure resulting in lesions which could be more readily detected by transcription coupled error prone DNA repair or highly inaccurate translesional DNA synthesis, resulting in the incorporation of wrong bases.

Another important feature of the tamoxifen DNA adducts investigated in this study is the low adduct levels required to induce mutagenesis above background, compared to published reports for carcinogen adducts. Previously, mutation frequencies similar to those reported here (i.e. around 1×10^{-3} with 0.05 4-hydroxytamoxifen adducts per plasmid), have been reported for *supF* plasmid containing acetylaminofluorene (AAF) (Mah, *et al.*, 1991), BPDE (Yang, *et al.*, 1987), or 1,6-dinitropyrene adducts (Boldt, *et al.*, 1991), replicated in the same cell line (with the same repair proficiency), but containing between 20 and 460 times as many adducts per plasmid as the 4-hydroxytamoxifen. In our experiments, α-acetoxytamoxifen showed a similar mutagenicity, on an adduct per plasmid basis, as has been reported for BPDE (Yang, *et al.*, 1987).

Previously reported mutation spectra in Ad293 cells (Bigger, *et al.*, 1990; Juedes and Wogan, 1996), human lymphoblasts (Sikpi, *et al.*, 1991) and monkey kidney cells (Keyse, *et al.*, 1988) have shown that the GC→AT transition is the preferred spontaneous mutation. In this investigation GC→TA transversions are just as prevalent as GC→AT transitions in control plasmids, and GC→CG transversions are only slightly less so. Upon treatment, the GC→AT transition becomes the major

mutation for α-acetoxytamoxifen multiple substitutions and 4-OHtamQM single base substitutions. The GC→TA transversion becomes the preferred mutation in α-acetoxytamoxifen single base substitutions and 4-OHtamQM multiple substitutions. The induction of transition mutations by 4-OHtamQM could, potentially, be explained by the fact that rat liver microsomal activation of tamoxifen and 4-hydroxytamoxifen has been shown to induce production of 8-hydroxy-2'-deoxyguanosine in DNA (Ye and Bodell, 1996). Small adducts such as this could contribute to transition type mutations. Miscoding of the damaged DNA consistent with induction of the GC→TA transversion has been demonstrated for the dG-N^2-tamoxifen adduct in site-specifically modified oligonucleotides replicated *in vitro* (Shibutani and Dasaradhi, 1997). This mutation is commonly found for bulky aromatic DNA adducts, and is thought to be the result of misincorporation of adenine opposite the damaged base in order to facilitate polymerase by-pass of the bulky lesion (the A-rule, Boiteux and Laval, 1982; Schaaper, *et al.*, 1983; Sagher and Strauss, 1983). As well as misincorporation of A opposite the dG-N^2-tamoxifen adduct, misincorporation of G and, in some sequences, T was also shown to be possible, albeit at lower frequency. The occurrence of these misincorporation events *in vitro*, would lead to induction of GC→CG and GC→AT mutations, respectively. As well as being influenced by the sequence context of the adduct, the frequency of misincorporation of different bases was also affected by the choice of DNA polymerase.

For both treatments, all hotspots were at GC base pair sites, presumably as a result of adduct formation on deoxyguanosine. There was a noticeable preference for the hotspot site to be preceded and followed by a purine, particularly adenosine. Positions 155, 156 and 174 had hotspots in two or more of the spectra. Hotspots at

156 and 174 appeared in both multiple base substitution spectra and the hotspot at 156 was present in the 4-OHtamQM single spectra. Position 156 also showed as a hotspot in the control plasmid that was incubated with either acetonitrile or ethanol only. Whether the solvent caused this is not known although Lewis *et al* did see a hotspot at this position when grouping spontaneous mutations (Lewis, *et al.*, 2001). Single and multiple base substitutions have been presented on separate spectra because it has been suggested that these multiple mutations arise through a different mechanism to single base mutations (Courtemanche and Anderson, 1999).

The higher percentage of plasmids with multiple mutations induced by 4-OHtamQM compared to α-acetoxytamoxifen may point to a different mechanism of mutagenesis accounting for some of the mutations induced by the former. Similarly, the increased amount of both insertions and large deletions in plasmids treated with 4-OHtamQM compared to α-acetoxytamoxifen suggests that not all mutations induced by 4-OHtamQM are due to misreading of adducted bases, but may be due, in part, to the formation of cross-links between bases. Previous work has shown that the cross-linking agent mitomycin C, which reacts with deoxyguanosine at the *N*-2 position like tamoxifen, induces a comparable amount of deletions to 4-OHtamQM in Ad293 cells (34% of all mutations) (Maccubin, *et al.*, 1997) along with a large amount of GC→TA transversions. Mitomycin C is used as a treatment for bladder and rectal cancers due to its DNA cross-linking ability. The anticancer drug Melphalan, also causes DNA cross-linking, probably by *N-7* alkylation of two guanines on opposite DNA strands. When used to treat pZ189 plasmid containing the *supF* gene in Ad293 cells this also induced a large proportion of deletions (16-28%) (Wang, *et al.*, 1990). Metabolic activation of both tamoxifen and 4-hydroxytamoxifen in rat liver

microsomes result in the formation of dimers, possibly through a free radical mechanism (Jones, *et al.*, 1999). A possible explanation for the induction of large deletions with 4-hydroxytamoxifen derived adducts may therefore be the generation of free radical species resulting in the production of tamoxifen dimer adducts which could be in the form of inter or intrastrand cross links. It has previously been reported that multiple mutations correlate with increased levels of single strand breaks in plasmid DNA, either induced by treatment or during repair of induced lesions in repair competent cells (Seidman, *et al.*, 1987). The production of free radical species could therefore account for this increase in both multiple mutations and deletions, with treatment by 4-OHtamQM, via the induction of strand breaks.

Since the *supF* target gene is a double stranded DNA molecule it is not possible to determine absolutely which strand contained the mutagenic lesion. However, as tamoxifen does not induce significant amounts of damage at cytosine residues and it is known that the dG-N^2-tamoxifen adduct induces GC→TA mutations, it is probable that the majority of GC→TA mutations are due to misincorporation opposite a damaged G. Considering this, it is possible to review the mutation spectrum from the point of view of any possible strand bias, as the coding strand is more efficiently repaired than the non-coding strand (Chen, *et al.*, 1990; Inga, *et al.*, 1994). After treatment with 4-OHtamQM, the number of mutations presumably derived from damage to the transcribed and non-transcribed strands are almost the same (94 vs 103). After treatment with α-acetoxytamoxifen, there is less damage induced in the transcribed strand than the non-transcribed strand (72 vs 114). This may suggest that adducts derived from α-acetoxytamoxifen are repaired more efficiently than those formed by 4-OHtamQM in this cell line. This is in contrast to previous work from

this laboratory that has shown that tamoxifen adducts are removed from rat liver DNA with no detectable difference in the rates of repair of individual adducts (Carthew, *et al.*, 1995a; Martin, E.A, *et al.*, unpublished results). However, using an *in vitro* human nucleotide excision repair system, site-specific dG-N^2-tamoxifen adduct isomers in oligodeoxyribonucleotides have been shown to be differentially repaired (Shibutani, *et al.*, 2000b). Overall adducts were removed with a poor to moderate efficiency with the *cis*-forms being removed most efficiently. It is therefore likely that differences in the chemical structure and overall adduct profile will have a significant influence on the repair and consequently mutation spectrum of tamoxifen adducts in human cells.

4.4.2 Part C and D: Mutagenicity of plasmid treated with α-acetoxytamoxifen replicated in GM00637 normal cells or in GM04429 XPA cells

In this study the mutation spectra induced when α-acetoxytamoxifen treated plasmid was replicated in two cell lines, GM00637 repair proficient cells and repair deficient XP cells (GM04429), has been investigated. Replication of α-acetoxytamoxifen adducted plasmid resulted in an increase in mutation frequency above background control levels in both cell lines. Furthermore, mutation frequencies increased with plasmid adduct levels, as was the case when α-acetoxytamoxifen adducted plasmid was replicated in Ad293 cells. In all cases, the majority of mutations were base substitutions at GC pairs. For reasons that are unclear, the solvent control mutation frequency in these experiments was about 6-29-fold higher than was expected compared to the spontaneous mutation frequency of plasmid treated with water only.

When the ethanol treated control plasmid was replicated in Ad293 cells an increase in mutation frequency was observed compared to water control (33-fold increase). Replication in GM04429 cells of ethanol treated plasmid resulted in a mutation profile similar to the GM00637 published control (Myrand, *et al.*, 1996; Seidman, *et al.*, 1987), where plasmid was treated with TE buffer only. Mutations observed in TE buffer control replicated in GM04429 cells were reported by Myrand, *et al.* (1996), where there was a preference for GC→AT transitions (57%) over GC→TA transversions (23%). In the present study there is also a preference for GC→AT transitions (44%) over GC→TA transversions (19%) in GM04429 cells. The increase in mutation frequency by solvent treatment of plasmid may be due to the formation of ethanol-DNA adducts, but these do not result in an increase in transition mutations over buffered water controls.

In the control samples, and plasmid dosed with 10 μM α-acetoxytamoxifen, the most common base substitution was the GC→AT transition. Upon treatment with 50 μM α-acetoxytamoxifen, the most common mutation observed was the GC→TA transversion (the 25 μM dose cannot be compared due to the low number of colonies available for sequencing). In these experiments, the fact that the relative frequency of different types of base substitution at 50 μM α-acetoxytamoxifen was similar in both cell lines suggests that DNA repair did not greatly influence the induction of different types of mutation. However, the distribution of hotspots of damage did vary between the different cell types, showing that cellular background does influence mutagenic spectra (Kawanishi, *et al.*, 1998a&b; Levy, *et al.*, 1992; Mah, *et al.*, 1989&1991; Bredberg, *et al.*, 1886). In the single and tandem base substitution mutation spectra there were no common sites of mutation hotspots between the two cell lines.

Looking, however, at the multiple mutation spectra, there is a site (position 149) which is common. The proportion of multiple mutations does not seem to significantly change between the cell lines (between 14-29% in GM00637 cells and 9.5-33% in XP cells). It has been suggested that these multiple mutations arise through a different mechanism to single base mutations (Courtemanche and Anderson, 1999). Multiple mutations may arise during translesion DNA synthesis and involve an error-prone polymerase able to introduce a base opposite misinstructive or noninstructional DNA lesions and subject to subsequent misincorporation errors. The mechanism for multiple mutations is then independent of the nucleotide excision repair ability of the cells. Of course multiple DNA lesions on a plasmid could have induced multiple base substitutions, but at the concentrations used in this study, this would seem somewhat unlikely.

In this study we have been looking at relatively low levels of DNA damage. Our range of adduct levels of between 0.5 and 2 adducts per plasmid are much lower than doses previously reported for these types of experiments. Mah *et al* (1991) treated *supF* containing pZ189 shuttle vector with aminofluorene (8-40 adducts per plasmid) in Ad293 cells to give mutation frequencies in the region of 3-20 $\times 10^{-4}$. pSP189 plasmid was treated with aflatoxin B_1 by Levy *et al* (1992) (6-22 adducts per plasmid) in both XP12BE (GM04429) and GM00637 cells to give mutation frequencies in the ranges 6-26 $\times 10^{-4}$ and 3-8.3 $\times 10^{-4}$ respectively. The bulky adducts of 1,6-dinitropyrene (23-44 adducts per plasmid) gave mutation frequencies in the range of 18.6-43.2 $\times 10^{-4}$ in human Ad293 cells (Boldt, *et al.*, 1991). Our experiments yield comparable mutation frequencies (up to 14 $\times 10^{-4}$ and 19×10^{-4}, for XP and normal,

respectively) but at much lower doses, suggesting that the α-(deoxyguanosin-N^2-yl)-tamoxifen-DNA adduct is potentially very mutagenic.

It is possible to evaluate the contribution of repair to the mutagenicity of the α-(deoxyguanosin-N^2-yl)-tamoxifen-DNA adduct by comparing the mutation spectra for GM00637 'normal' and GM04429 XP repair deficient cells. The mutation frequencies do not differ appreciably between the cell lines. However, there is a difference in the distribution of both type and position of mutations. XP cells predominantly give GC→TA transversions whilst, except at the highest dose, normal cells give GC→AT transitions. These transitions, which are also seen in control plasmid, could be preferable or less detrimental to future cell survival than the transversions. Another difference between normal and XP cells is the increased level of deletions (both of a single base and multiple bases). The XP cells are lacking the *XPA* damage recognition protein resulting in DNA lesions not being removed. When the cells replicate these damaged pieces of DNA, the polymerase could, conceivably, detach from the template strand at the point of damage and re-attach further along the DNA on a clearer patch resulting in variable lengths of DNA being lost. When Aflatoxin B_1 was used in the *supF* assay in XP cells this large number of big deletions was not seen (Levy, *et al.*, 1992). 3-Nitrobenzanthrone in XP cells induces a higher proportion of single base deletions in comparison to normal cells, although a lower proportion of larger deletions (Kawanishi, *et al.*, 1998a). Acrolein treated plasmid replicated in normal human fibroblasts (W138-VA13) caused 20% of all mutations to be deletions (Kawanishi, *et al.*, 1998b), although the number of deletions from spontaneous mutations was just as high (19%). These studies all used higher doses than were used in this study so it seems that even when cells are DNA repair

proficient; if you insult the DNA enough, or damage is irreparable, adducts are not removed resulting in the induction of deletions.

Comparing the repair proficient GM00637 cells with the repair deficient, equivalent XP cell line, it is of interest to note that the number of mutations presumably derived from the transcribed and non-transcribed strands in the GM00637 cells are 44 and 57, respectively, compared to 36 and 23 in the XP cells. The apparent increase in the relative number of mutations in the transcribed strand in the XP cells could be due to the loss of transcription coupled repair in the XP cell line.

4.4.3 Summary

There is some evidence that tamoxifen adducts are formed in tissues of women taking this drug therapeutically (Hemminki, *et al.*, 1996 & 1997; Shibutani, *et al.*, 1999 & 2000a). Although these adducts may be formed at low levels, it has been shown in this study that tamoxifen DNA adducts may be highly mutagenic. In particular, adducts formed by 4-OHtamQM in the *supF* gene are, when replicated in human Ad293 cells, more mutagenic than those formed by α-acetoxytamoxifen (the major tamoxifen-DNA adducts). The two treatments induce markedly different mutation spectra and mutation types. One could conclude that the 4-hydroxytamoxifen metabolite of tamoxifen has potential to cause serious mutagenic damage to DNA. There is also evidence that the cellular background is important to the distribution of tamoxifen induced mutations in DNA. α-Acetoxytamoxifen adducts replicated in three different cell lines induced three different mutation distributions. Whether the genotoxicity and

mutagenicity of tamoxifen induced DNA adducts is a contributing factor in the induction of endometrial cancer in women remains to be seen.

Chapter 5

Development of a novel site-selective mutagenesis assay using LC-MS

5.1 Introduction

5.1.1 Development of a site specific mutagenesis assay using LC-MS

The earlier chapters of this thesis have discussed the effects of carcinogens and mutagens on the induction of post replicative DNA mutations in the *supF* forward mutation system. This assay is extremely valuable for quantifying the relative mutagenicity of different chemicals, by enabling comparisons of mutation frequencies. In addition it provides a qualitative insight into the types and distribution of mutations in the *supF* gene. However, this is not a biologically important gene, just a marker for mutagenesis; to identify which mutations could be caused by these various chemicals if humans were exposed to them. In the case of tamoxifen (Chapter 4) it has been shown that mutations are preferentially induced at guanines and that different deoxyguanosine tamoxifen DNA adducts cause different mutations; GC→TA transversions by α-(N^2-deoxyguanosinyl)-tamoxifen and GC→AT transitions by α-(N^2-deoxyguanosinyl)-4-hydroxytamoxifen. However, these studies do not identify exactly which isomer of these adducts is responsible for which mutational event. Furthermore, whilst the results indicate GC→TA transversions or GC→AT transitions are important these experiments do not provide information on whether tamoxifen derived DNA adducts cause mutations in human genes which might be involved in tamoxifen induced endometrial carcinogenesis. The aim of the work described in this chapter is to develop a new mutagenesis assay that can be used to identify potential important genes and investigate the site selectivity of tamoxifen adduction and mutations using relevant biological DNA sequences. This assay will involve the use of small deoxyoligonucleotides containing a single, pure, DNA adduct

which will be employed in investigations of site selective mutagenesis in a variety of important cancer related genes, i.e. *p53, K-ras*, etc.

5.1.2 Factors affecting mutagenic potential of a genotoxic chemical

Many structural and biological features influence the mutagenic potential of a genotoxic chemical. The chemical structure of the DNA adduct and even stereochemistry has a major affect on repair and mutagenesis (Shibutani, *et al.*, 2000b). Furthermore, the DNA sequence surrounding an adduct can have a profound influence, probably due to effects on the 3-dimensional structure of the adduct and resulting DNA distortions. Changes in sequence context even at bases distant to the site of adduction can have effects on the biological outcomes (Levy, *et al.*, 1996 a&b). Therefore, the mutagenicity of a particular adduct may often be dependent on the gene it is in. It is therefore important to be able to study the repair and mutagenicity of individual adducts in numerous sequence contexts, preferably in genes relevant to human cancer.

5.1.3 Background on sequence selective mutagenesis

The study of the role of sequence in base modification was started in the 1980s in terms of sequence selectivity for mutational events. The mutational events studied were generally produced by environmental agents, such as UV exposure, alkylating agents and aromatic amines, or spontaneously (Singer and Grunberger, 1983). It has since become clear that such events are not totally random but tend to predominate at

specific sites in nucleic acids, as shown in Chapters 2-4 for *supF* mutation spectra. This type of investigation led to the idea of `targeted' mutation, which was often related to specific cellular changes in function.

As well as an interest in how base sequence affected modification it is also interesting to investigate whether the base sequences flanking damage could also influence replication (Toorchen and Topal, 1983; Petruska and Goodman, 1985; Singer, *et al.*, 1989) and repair (see for example: Burns, *et al.*, 1986; Dolan, *et al.*, 1988; Jones, *et al.*, 1987; Seeberg, and Fuchs, 1990). Significant progress in these research areas was accelerated when it became feasible to design and construct synthetic oligonucleotides with site-directed modified bases (Singer and Essigmann, 1991). In addition, it became generally accepted that nucleic acid sequence and structure are closely linked. Changes in both primary and secondary structures resulting from neighbouring bases were presumed to be one of the important factors affecting replication and repair, both qualitatively and quantitatively.

When DNA synthesizers became widely available in the mid 1980s, it was possible to design oligonucleotides of virtually any sequence containing normal or modified bases for which phosphoramidites could be made. With this powerful tool, a new era began in the study of the effects of chemical modification on DNA structure, replication and repair.

One fact that has emerged is that the sequence context surrounding a lesion has an influence on the rate and extent of enzymatic repair. Among the lesion types or modifications that have been studied for repair influenced by neighbouring sequences are *N*-acetyl-2-aminofluorene (AAF) (Seeberg, and Fuchs, 1990; Mekhovich, et al., 1998), *N*-2-aminofluorene (AF) (Mekhovich, *et al.*, 1998), benzo[*a*]pyrene diol

epoxide (BPDE) (Wei, *et al.*, 1995), UV photoproducts (Svoboda, *et al.*, 1993; Tornaletti and Pfeifer, 1994), 1,N^6-ethenoadenosine (εA) (Hang, *et al.*, 1998a), *p*-benzoquinone derivatives (Hang, *et al.*, 1998b), alkyl bases (Topal, *et al.*, 1986; Dolan, *et al.*, 1988; Bender, *et al.*, 1996; Delaney and Essigman, 1999), base mismatches (Jones, *et al.*, 1987; Ullah, *et al.*, 1996; Saparbaev and Laval, 1999), uracil (Eftedal, *et al.*, 1994) and apurinic/apyrimidinic (AP) sites (Sági, *et al.*, 1999). The repair systems used for these lesions include enzymes from base excision repair, nucleotide excision repair (NER), mismatch repair, O^6-alkylguanine-DNA alkyltransferases (AGT) and photolyase. In addition, the sequence dependence of the special 'repair' activity (proof-reading) of DNA polymerases was also studied (Petruska and Goodman, 1985; Bloom, *et al.*, 1994). Both *in vivo* and *in vitro* studies have been reported.

Data from *in vivo* modification and repair can be obtained from natural DNA sequences (*lacI, p53,* etc) or from foreign sequences, such as *supF* (Seidman, *et al.*, 1985) or synthetic deoxyoligonucleotides, and gives an insight into factors influencing both events. The general approach is to study a specific sequence such as a gene [i.e. *lacI*; (Burns, *et al.*, 1987), *PGK1*; (Gao, *et al.*, 1994), *HPRT*; (Bol, *et al.*, 1998) and *p*53; (Tornaletti and Pfeifer, 1994)] or a sequence in a vector in the cell [i.e. *supF* assay (McLuckie, *et al.*, 2002)]. DNA is treated with a chemical to give a mutation spectrum. Modification depends on both the specificity of the compound of interest and the base composition of a given sequence. Consequently, repair can take place at these chemically modified sites and sequences. Some *in vitro* experiments on sequence specificity utilise a biologically relevant partial sequence from a genome. Sequences can be designed and synthesised to test the effects on repair of specific factors such as sequence context, adduct type, position and thermodynamics.

The different approaches discussed above have their limitations: particularly *in vivo*, it is generally not possible to study the effect of neighbouring bases alone, but rather it is usually represented as a position effect which includes the influence of immediate sequence context. Another important aspect of the *in vivo* studies is that a given adduct can be repaired by more than one pathway. For example, O^6-alkylguanine can be repaired by at least two alternative pathways in *E.coli*: the *Ada* and *Ogt* methyltransferases and the NER pathway (Bronstein, *et al.*, 1991; Samson, *et al.*, 1988; Voigt, *et al.*, 1989). The involvement of chromatin structure *in vivo* is also an important factor in repair, as shown by Li and Smerdon (1999). Interpretation of repair data obtained under these different circumstances can be ambiguous and desired changes in the experimental design are not necessarily possible.

Extensive work on relating sequence dependence and mutation has been done by several groups using chemical modification of genomic DNA, followed by determination of the mutation spectra (Koffel-Shwartz, *et al.*, 1984; Burns, *et al.*, 1987; Cariello and Skopek, 1993; Kamiya, *et al.*, 1995; Bol, *et al.*, 1998). These data were among the first used to substantiate the concept of 'hot spots', i.e. for a given compound, there was site specificity for DNA modification. There were also indications that selective repair may be a factor which affects persistence of the adduct at specific sites in DNA when different repair backgrounds were used. For example Burns *et al.* (1986) reported the mutagenesis and selectivity of ethyl methane sulphonate in different repair backgounds in *E.coli* (Uvr^+ versus $UvrB^-$ strains). According to the authors, ethyl^6-dG lesions adjacent to AT base pairs are better repaired than those with GC pairs at either the 5'- or 3'-side.

Several groups in the mid 1990s examined the *in vivo* repair rates along a specifically modified DNA fragment in a more direct and sensitive way by using the ligation-mediated PCR technique (Wei, *et al.*, 1995; Tornaletti and Pfeifer, 1994; Gao, *et al.*, 1994; Ye, *et al.*, 1998). For example, Wei *et al.* (1995) used BPDE modification in the *HPRT* gene of human fibroblasts. The repair rate was markedly different from site to site over a time period of 0–30 h, as measured by the percentage of adduct remaining. Tornaletti and Pfeifer (1994) investigated the repair of cyclobutane pyrimidine dimers along the *p53* gene in human cells irradiated with UV light. It was found that repair rates at different positions were also highly variable, ranging from 70–95% repaired to almost an absence of repair after 24 h. Interestingly, slow repair was observed at seven of eight positions which are frequently mutated in skin cancer (Tornaletti and Pfeifer, 1994). This is one of those few examples that clearly document a correlation between repair and the occurrence of mutation hot spots. These experiments show that quantitation of adduct site and repair background can be used to study *in vivo* repair sequence specificity. The data discussed above support the hypothesis that selectivity of repair of mutational events is an important factor in specificity of mutation.

5.1.4 Site-specific mutagenesis assays

Many methods have been developed to investigate mutagenesis using synthetic oligonucleotides containing single DNA adducts. One such assay, developed by Delaney and Essigmann (1999) uses viral genomes containing certain base sequences incorporating the adduct of choice which are transfected into *E.coli*. Restriction digestion and radioactive one-dimensional thin layer chromatography (TLC) can be used to identify which mutations are preferentially induced by DNA lesions in the site

specific sequences used. This assay has been used to investigate O^6-methylguanine and 7,8-dihydro-8-oxoguanine lesions (Delaney and Essigmann, 2001; Henderson, *et al.*, 2002), both of which are potent sources of replication errors *in vivo*. Although methods like this increase our understanding of the site selectivity and mutagenicity of DNA lesions in *E.coli* in single stranded DNA, they do not tell us much about mammalian systems.

5.1.5 A novel site-selective mutagenesis assay using LCMS

A method has been developed (Laken, *et al.*, 1998) which uses mass spectrometric analysis along with PCR based amplification of short DNA fragments to investigate single nucleotide polymorphisms (SNP's). The method uses a restriction enzyme (Bpm1) and specifically designed primers to yield small (7-20 basepairs) DNA fragments which are analysed by mass spectrometry. Any differences in mass will be due to differences in sequence, i.e. adenines can be resolved from thymidines due to the 9 Da difference in mass. This method can be adapted to be used as a site selective mutation assay. Synthetic deoxyoligonucleotides can be inserted into the *supF* gene of the pSP189 plasmid (Seidman, *et al.*, 1985; Parris and Seidman, 1992). When the plasmid has been transfected into, and recovered from, human cells the presence of the synthetic insert will act as a frameshift mutation, thus inactivating the *supF* gene, resulting in the production of white mutant colonies (Figure 5.1). PCR followed by digestion with Bpm1 yields small (16 basepairs) double stranded deoxyoligonucleotides which can then be analysed by mass spectrometry (Figure 5.2.).

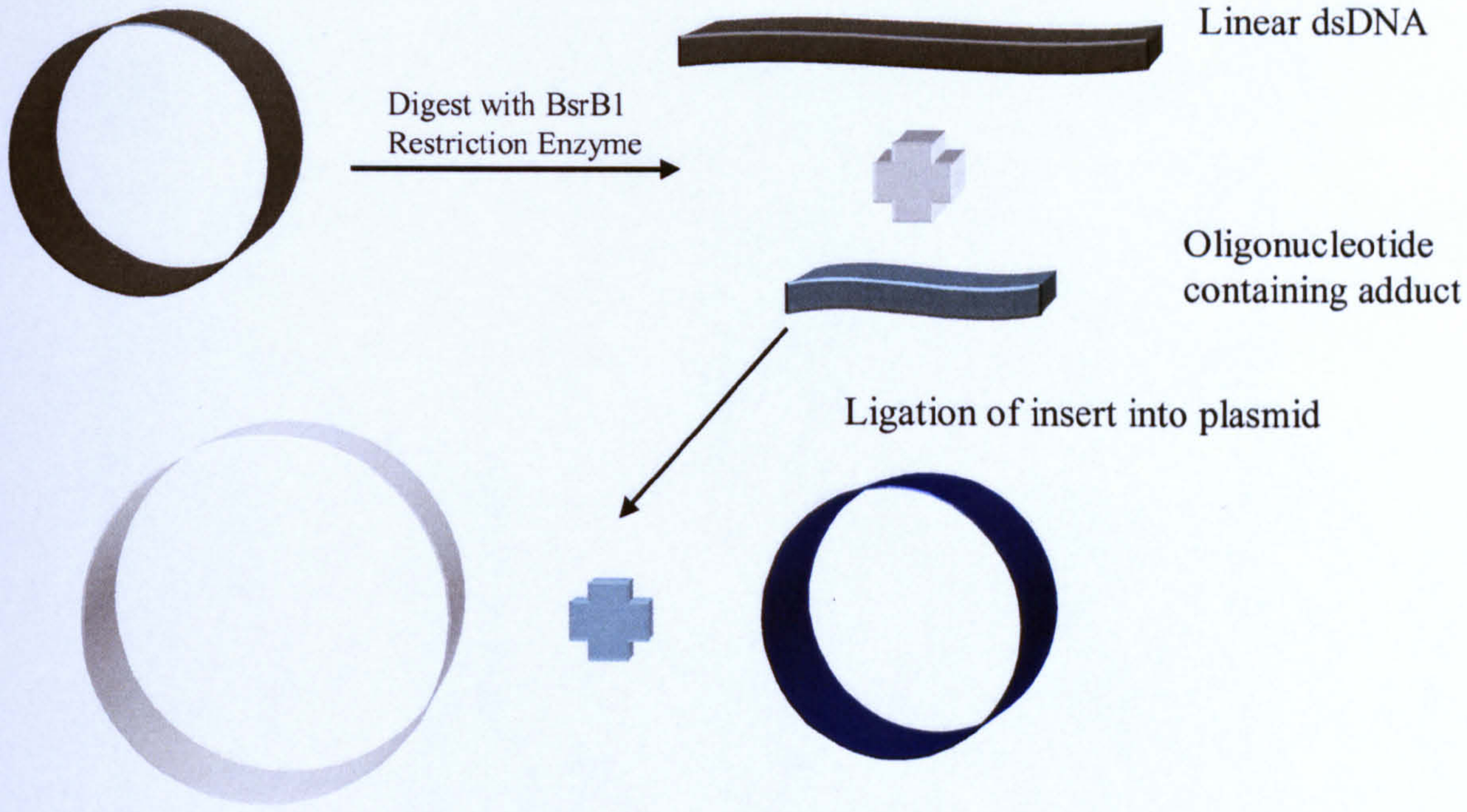


Figure 5.1 Insertion of double stranded deoxyoligonucleotides results in a frameshift mutation in the *supF* gene. Correct ligation of the insert results in the growth of white colonies after replication in human cells and transformation in *E.coli*.

The deoxyoligonucleotide used to validate this assay was designed so that it can be treated with a chemical such as α-acetoxytamoxifen, which preferentially binds to deoxyguanosine bases. The deoxyguanosine is flanked by deoxycytidine and thymidine bases which are much less chemically reactive with α-acetoxytamoxifen. Treatment of the single stranded deoxyoligonucleotide followed by HPLC purification will yield a mono-adducted insert which can be used to investigate the mutagenicity of the α-(deoxyguanosin-N^2-yl)-tamoxifen DNA adduct (see Chapter 4: Osborne, *et al.,* 1996; Rajaniemi, *et al.,* 1999).

Figure 5.2 (overleaf) Illustrates an outline of the PCR / restriction digestion steps for the site selective mutation assay.

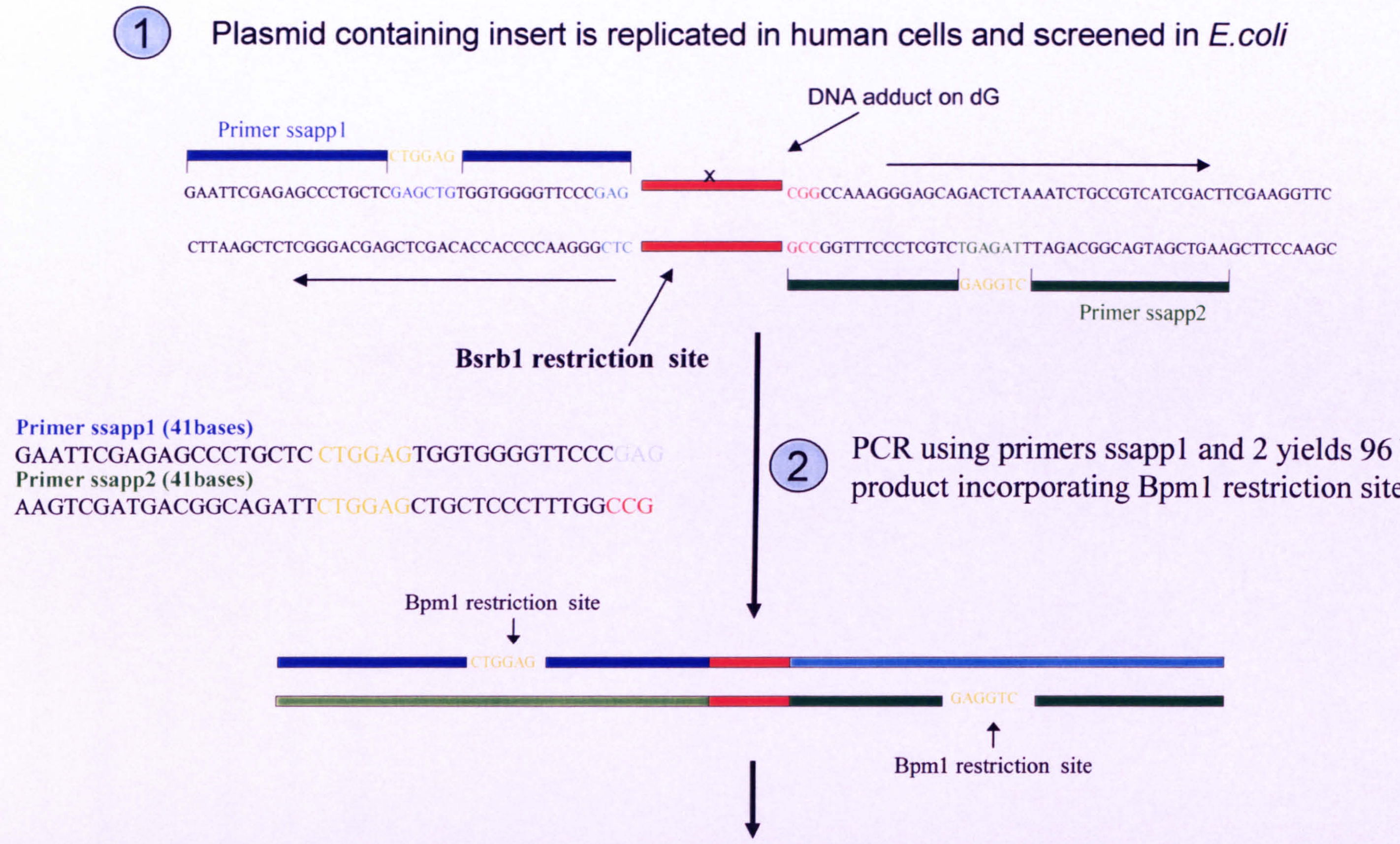

1
Plasmid containing insert is replicated in human cells and screened in E.coli
DNA adduct on dG
Primer ssapp1
CTGGAG
X
GAATTCGAGAGCCCTGCTCGAGCTGTGGTGGGGTTCCCGAG
CGGCCAAAGGGAGCAGACTCTAAATCTGCCGTCATCGACTTCGAAGGTTC
CTTAAGCTCTCGGGACGAGCTCGACACCACCCCAAGGGCTC
GCCGGTTTCCCTCGTCTGAGATTTAGACGGCAGTAGCTGAAGCTTCCAAGC
GAGGTC
Primer ssapp2
Bsrb1 restriction site
Primer ssapp1 (41bases)
GAATTCGAGAGCCCTGCTC CTGGAGTGGTGGGGTTCCCGAG
Primer ssapp2 (41bases)
AAGTCGATGACGGCAGATTCTGGAGCTGCTCCCTTTGGCCG
2
PCR using primers ssapp1 and 2 yields 96 base pair product incorporating Bpm1 restriction sites
Bpm1 restriction site
CTGGAG
GAGGTC
Bpm1 restriction site
3
Digestion with Bpm1 restriction enzyme yields replicated insert
(double stranded 16 mer)
4
Mass analysis shows whether insert has been repaired (dG) or mutated to dA, dT or dC.

Figure 5.2

It has recently been shown that tamoxifen DNA adducts can be inserted into deoxyoligonucleotides by phosphoramidite chemical synthesis (Laxmi, *et al.*, 2002). It should be possible once this assay is validated to investigate the site selectivity of tamoxifen DNA adducts using different DNA sequences, such as with multiple deoxyguanosines. Furthermore, any sequence or DNA adduct can be investigated as long as a DNA-adduct phosphoramidite is available for insertion into the synthetic deoxyoligonucleotide insert.

5.2 Materials and Methods

5.2.1 Materials

All chemicals were from Sigma-Aldrich, Poole, Dorset, UK unless otherwise stated.

Methods

All methods specific to this chapter are described below. For any other procedures refer to Chapter 2.

5.2.2 Preparation of deoxyoligonucleotides

Deoxyoligonucleotide inserts (INS3A and INS3B, where G is the proposed site of adduction), mass spectrometry standards (INS3Y through to INS3ZC), and site selective assay PCR primers (ssapp) 1 and 2 were prepared:

INS3A	CTT CCT CGC TCT TC
INS3B	GAA GAG UGA GGA AG
INS3Y	GAA GAG CGA GGA AGC T
INS3Z	CTT CCT CGC TCT TCC G
INS3YT	GAA GAG TGA GGA AGC T
INS3ZA	CTT CCT CAC TCT TCC G
INS3YA	GAA GAG AGA GGA AGC T
INS3ZT	CTT CCT CTC TCT TCC G
INS3YG	GAA GAG GGA GGA AGC T
INS3ZC	CTT CCT CCC TCT TCC G

ssapp1 GAA TTC GAG AGC CCT GCT CCT GGA GTG GTG GGG TTC CCG AG

ssapp2 AAG TCG ATG ACG GCA GAT TCT GGA GCT GCT CCC TTT GGC CG

The oligonucleotides were prepared by PNACL, Leicester (containing 5' phosphate groups). The optical density of the supplied oligonucleotides was measured at 260 nm. Prior to use the oligonucleotides were desalted by precipitation using 5 µl 3 M sodium acetate and 100 µl ethanol per optical density unit (ODU). The oligonucleotides were then briefly vortexed and incubated at -20°C overnight. The oligonucleotides were centrifuged for 5 min at 14000 rpm at 4°C and the supernatant removed. The pellets were washed with ethanol (100 µl per ODU) and re-centrifuged, as before. The final supernatant was removed and the oligonucleotides dried by vacuum centrifugation (Savant Industries INC., Farmingdale, NY., USA). The desalted oligonucleotides were dissolved in 100 µl sterile water and the concentration was calculated based on the optical density at 260 nm. The inserts were diluted to aliquots of 30 pmol with tissue culture grade water and stored at -20°C prior to use. The PCR primers were diluted to 50 pmol/ µl with tissue culture grade water and stored at -20°C prior to use. Mass spectrometry standards were stored dry at -20°C prior to use.

5.2.3 Annealing of INS3A and INS3B to form INS3

INS3A and INS3B (30 pmol) aliquots were combined, mixed, and then dried by vacuum centrifugation (Savant Industries INC., Farmingdale, NY., USA). INS3 was redissolved in 5 µl tris-EDTA buffer, pH 8.0 and allowed to anneal at room

temperature (20-25°C, 2 h). INS3 was stored at 4 °C prior to being ligated into pSP189 plasmid.

5.2.4 Digestion of pSP189 plasmid with Bsrb1 restriction enzyme

pSP189 Plasmid (10 μg, approx 3 pmol) was digested with Bsrb1 restriction enzyme (New England Biolabs (UK) Ltd., Hitchin, UK) (20 U) in NEBuffer 2 (50 mM sodium chloride, 10 mM Tris-HCl, 10 mM magnesium chloride, 1 mM dithiothreitol {pH 7.9 at 25°C}) in a total volume of 50 μl for 2 h at 37°C. Restriction enzyme was removed using Micropure EZ enzyme removers (following manufacturer's protocol; which involves centrifugation aided filtration) (Millipore Corporation, Bedford, MA, USA). The DNA was precipitated with 2 M sodium acetate (10 μl) and ice-cold ethanol (800 μl). The DNA samples were pelleted by centrifugation using a bench top centrifuge (Hettich, Germany), supernatants removed and pellets washed with 70% ice-cold ethanol followed by 100% ice-cold ethanol. Pellets were stored dry at -20°C prior to ligation.

5.2.5 Ligation of INS3 into pSP189 plasmid

Digested pSP189 plasmid was re-suspended in 10 μl 2× T4 DNA ligase buffer (New England Biolabs). To this DNA buffer mixture was added double stranded insert DNA (INS3, 30pmol) and T4 DNA ligase (2000 U). The total reaction volume was diluted to 20 μl with sterile water. Ligation took place at 16°C for 6 h. DNA ligase enzyme was removed using Micropure EZ enzyme removers (Millipore Corporation,

Bedford, MA, USA). DNA was precipitated as above and re-dissolved in 20 µl tris-EDTA buffer, pH 8.0 and stored at -20°C.

5.2.6 Transfection and transformation

Transfection of plasmid into Ad293 cells, recovery of plasmid and transformation of *E.coli* MBM7070 was carried out as described previously in Chapters 2 and 3.

5.2.7 Screening for mutant colonies

Aliquots of transformed E.coli MBM7070 were spread on agar plates (25 µg/ml IPTG, 75 µg/ml X-gal, and 100 µg/ml ampicillin) and grown overnight (16 h, 37°C) Any white colonies result from INS3 being ligated into the *supF* gene of pSP189 plasmid. White colonies were picked and grown to amplify the level of plasmid. Overnight cultures were pelletted and stored at -20°C prior to use.

5.2.8 Polymerase chain reaction (PCR) of MBM7070 E.coli containing pSP189 plasmid incorporating insert INS3

PCR master mix (25 µl, Abgene, Epsom, Surrey), tissue culture grade water (22 µl), ssapp1 (1 µl, 50 pmol), ssapp2 (1 µl, 50 pmol) and 1 ml MBM7070 *E.coli* was introduced into a 200 µl thin walled PCR tube and subjected to the following PCR program using a Tetrad thermal cycler (MJ Research, Inc.,Waltham, MA, USA): 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 60 s, final extension at 72°C for 5 min, then cooled to 4°C (optimised method). Final

concentrations in each tube were; 1.5 U *Taq* DNA polymerase, 75 mM Tris-HCL (pH 8.8 @ 25°C), 20 mM ammonium sulphate, 2 mM magnesium chloride, 0.01% (v/v) Tween 20, 0.2 mM each of dATP, dCTP, dGTP and dTTP. *Taq* DNA polymerase was removed after the reaction using Micropure EZ enzyme removers (Millipore Corporation, Bedford, MA, USA). Samples were then stored at -4°C until required.

5.2.9 Digestion of PCR products with Bpm1 restriction enzyme

The 96 basepair PCR products were digested with Bpm1 restriction enzyme (New England Biolabs (UK) Ltd., Hitchin, UK) (20 U) in NEBuffer 3 (100 mM sodium chloride, 50 mM Tris-HCl, 10 mM magnesium chloride, 1 mM dithiothreitol {pH 7.9 at 25°C}) in a total volume of 50 µl for 16 h at 37°C. Restriction enzyme was removed using Micropure EZ enzyme removers (Millipore Corporation, Bedford, MA, USA). The DNA was precipitated by the addition of an equal volume isopropanol and 10% 3 M sodium acetate. The PCR products were incubated at -20°C for 30 mins then pelleted by centrifugation using a bench top centrifuge (Hettich, Germany). The supernatant was removed and the pellets washed 2 times in 70% ice-cold ethanol followed by 2 times in 100% ice-cold ethanol. The pellets were stored dry at -20°C prior to analysis by LC-MS.

5.2.10 Analysis of double stranded deoxyoligonucleotides by HPLC coupled to electrospray mass spectrometry (LC-MS)

Mass spectrometry was carried out on a Micromass BioQ instrument (Micromass, Manchester, UK) equipped with a pneumatically assisted electrospray source. The

instrument was tuned using deoxyoligonucleotide standards (INS3Y/Z) which were infused using a Harvard Apparatus model 22 syringe pump (Harvard Apparatus, Edinbridge, UK) at 10 μl/ min. Analysis was carried out in negative ion mode with a source temperature of 110°. Samples were introduced into the source via a Varian 9012 LC pump (Varian, Walton-on-Thames, Surrey, UK) using an isocratic system with a flow rate of 20 μl/min. The mobile phase consisted of 80% 0.4 M hexafluoro-2-propanol in methanol. Samples (10 μl) were injected onto a 15 cm x 0.8 mm Vydac C_{18} column (Phenomenex, Macclesfield, Cheshire, UK) and a mass scan was carried out in the range 600-1300 Da/2sec for 15 min. Mass spectra from electrospray ionization sources showed multiple $[M\text{-}nH]^{n-}$ ion peaks. The mass spectrometer software can extrapolate the data from these charged peaks to a single mass corresponding to the non charged molecular mass. This is known as deconvolution to zero charge.

5.3 Results

The deoxyoligonucleotide CTT CCT CGC TCT TC (Insert 3A, INS3A) was designed to validate the site selective mutation assay as it has a single deoxyguanosine base flanked by deoxycytosines and thymidines. The insert could be treated with a selective mutagen, such as α-acetoxytamoxifen which preferentially binds to guanine, to form dG-DNA adducts. However, a non-treated control insert was used in the development and validation of the assay which is described in this chapter. The double stranded deoxyoligonucleotide insert INS3, was ligated into plasmid which had been digested overnight with Bsrb1 restriction enzyme to yield blunt ends in the sequence GAG^CGG. Correctly ligated inserts result in the introduction of a 'mutation' in the *supF* gene. The plasmid was transfected into human cells where a site selective DNA adduct (when present) can be repaired or mutated. The plasmid was recovered as described previously (Chapter 2) and screened in MBM7070 *E.coli* (Chapter 2). When grown on ampicillin, X-gal, and IPTG plates any plasmids with correctly ligated inserts resulted in mutant white colonies. These white colonies were picked and grown as 50 ml cultures to amplify the plasmid, which can then be analysed for any sequence changes.

Recovered plasmid samples were sequenced to check that the inserts had been properly ligated, as shown in Figure 5.3. Bacterial samples containing the plasmid and inserts were used for further development of the PCR amplification and Bpm1 digestion methods. PCR was carried out followed by digestion with Bpm1 to yield small (16 basepair) double stranded DNA fragments.

Insert 3A: CTTCCTCGCTCTTC

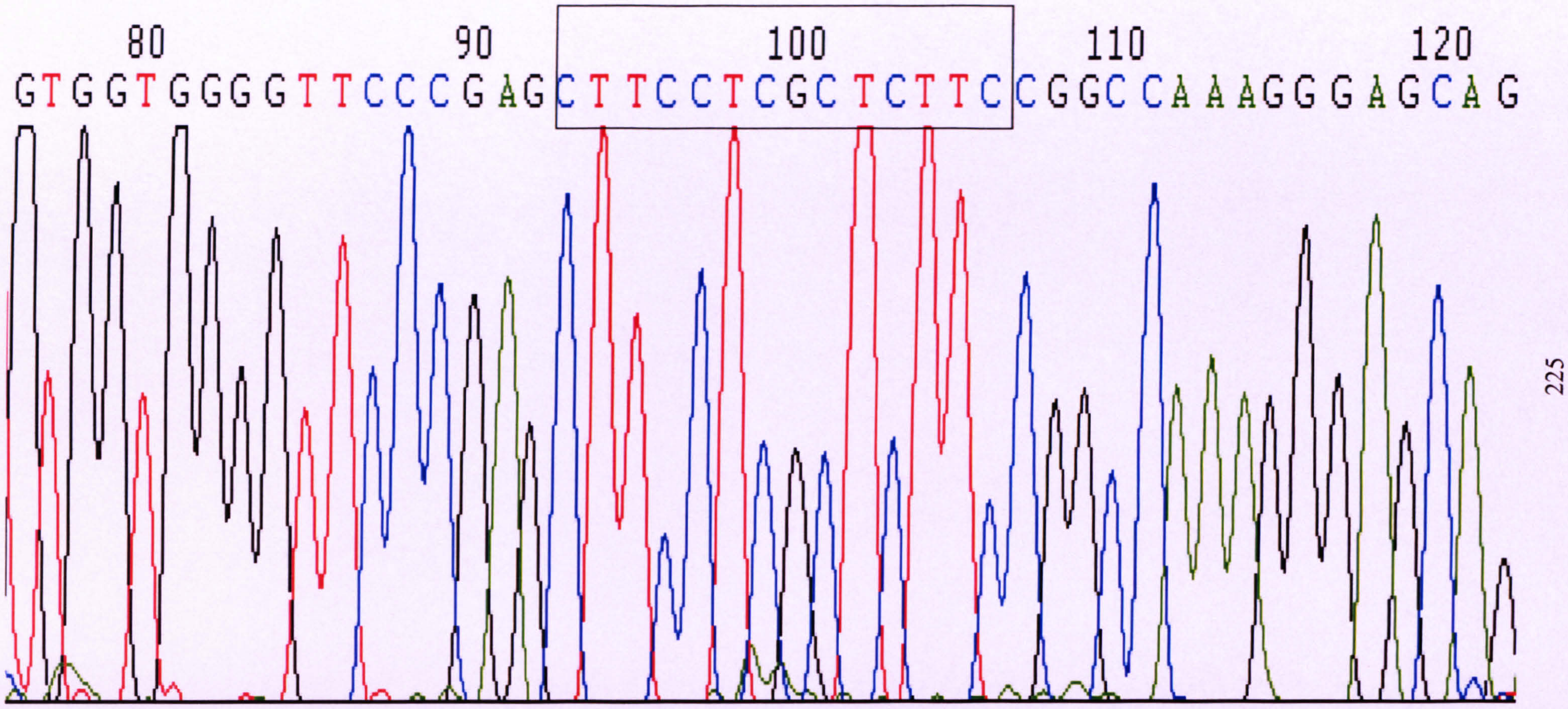


Figure 5.3. Plasmid containing INS3 deoxyoligonucleotide insert.

A PCR method which was already used in our laboratory was chosen to begin with. This method worked well, a variation was made which did not significantly increase yield (Figure 5.4.), and consequently this method was adopted for all future experiments.

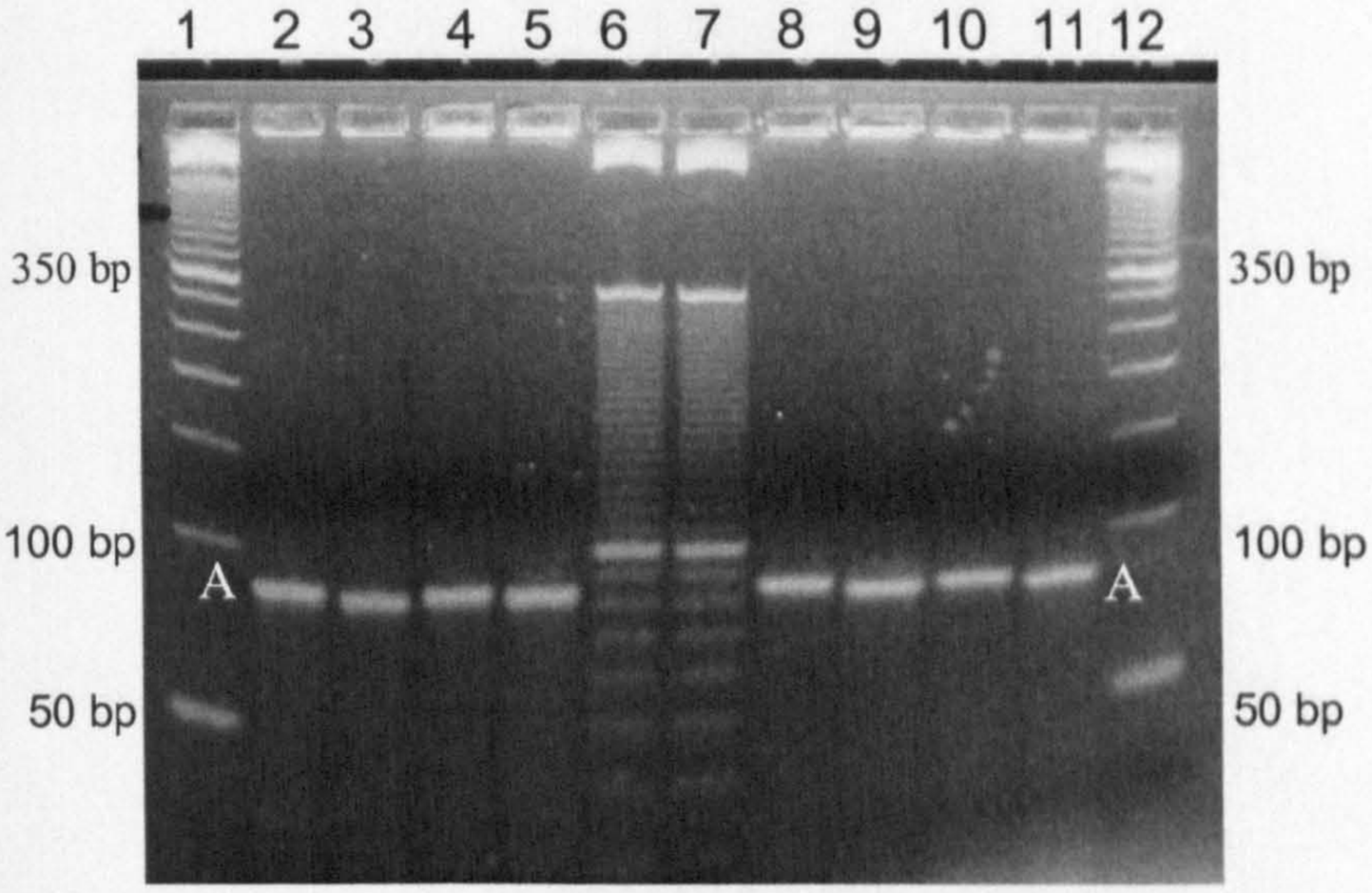


Figure 5.4 PCR method development. Illustration of a 4% agarose gel (70 V, 30 min) showing the 96 basepair (bp) PCR product (A). Lanes 2-5 represent separate reactions using 30 cycle PCR method and lanes 8-11 separate reactions using the 45 cycle method. Average intensity for 30 cycle method and 45 cycle method were 207 units and 197 units, respectively. Lanes 1, and 12 contain 50 bp DNA ladders, 6 and 7 contain 10 bp DNA ladders.

Figure 5.4 also illustrates that all of the available primers are used in the reaction (no bands lower on the gel) which would complicate further analysis, both by electrophoresis and mass spectrometry. The two PCR primers each contain a short 6 base sequence which is non-complementary to the replicated plasmid (Figure 5.2). In this way each primer is designed to insert a new Bpm1 restriction site into the 96

basepair double stranded PCR product. Digestion with Bpm1 therefore yields a 16 basepair product, which includes the original target sequence, which can be analysed for mutations. Figure 5.5 shows a gel illustrating the digestion of the 96 basepair double stranded PCR product to yield the desired 16 basepair double stranded product and two 40 basepair double stranded products. Incomplete digestion results in an extra band of 56 basepairs. The small 16 basepair product cannot be seen on this gel.

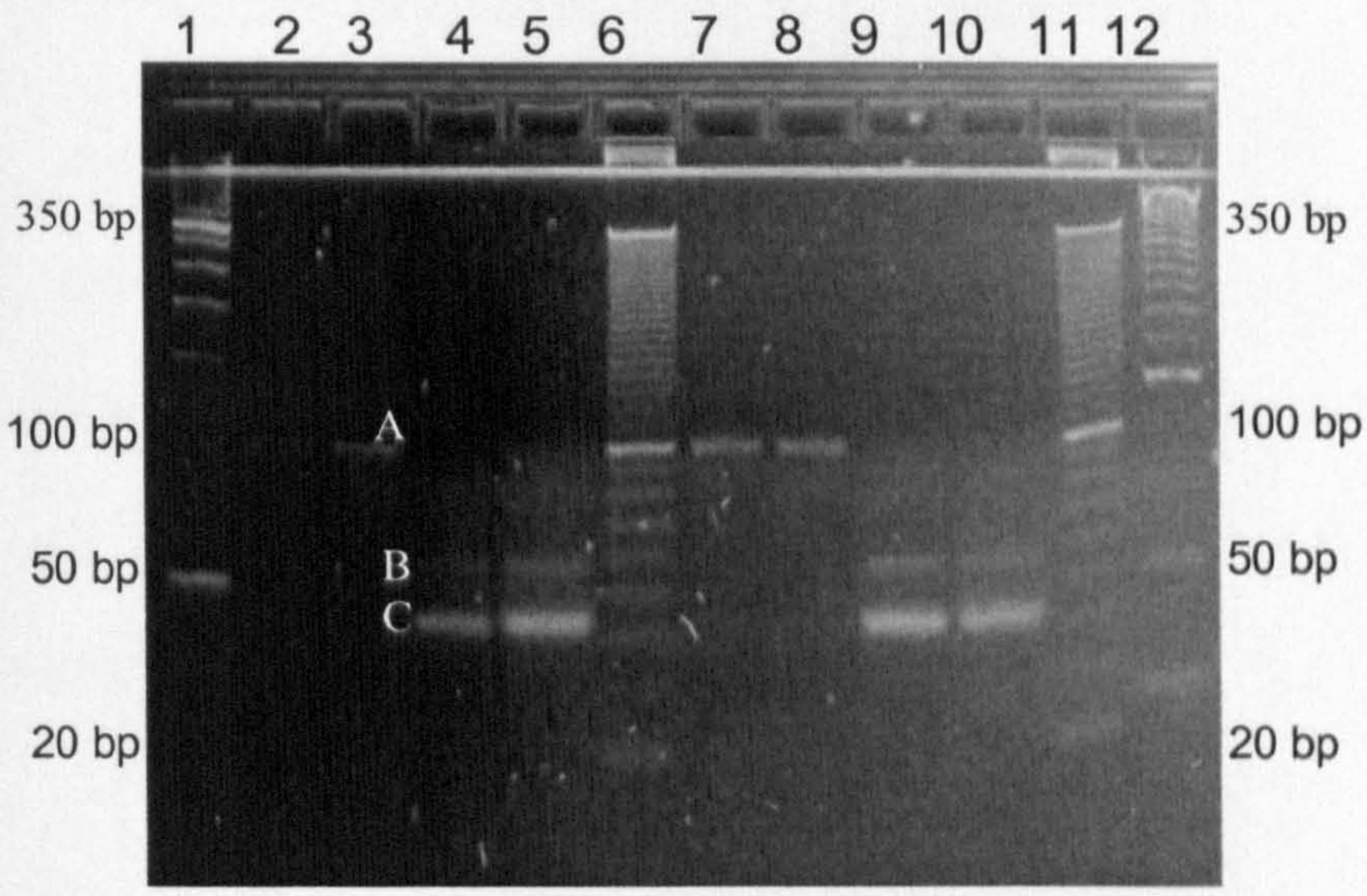


Figure 5.5 Digestion of 96 bp PCR product (A) yields 2 x 40 bp fragments (C), and a 16 bp fragment which is not visible. Band B corresponds to partially digested product containing 40 bp and 16 bp fragments. Lane 1 contains 50 bp ladder, 6 and 11 contain 10 bp ladder, 12 contains 25 bp ladder. Two separate PCR reactions are shown. Lanes 2, 3, 7, and 8 contain non-digested PCR product. Lanes 4, 5, 9 and 10 show digested PCR product. (4% agarose, 70 V, 30 min).

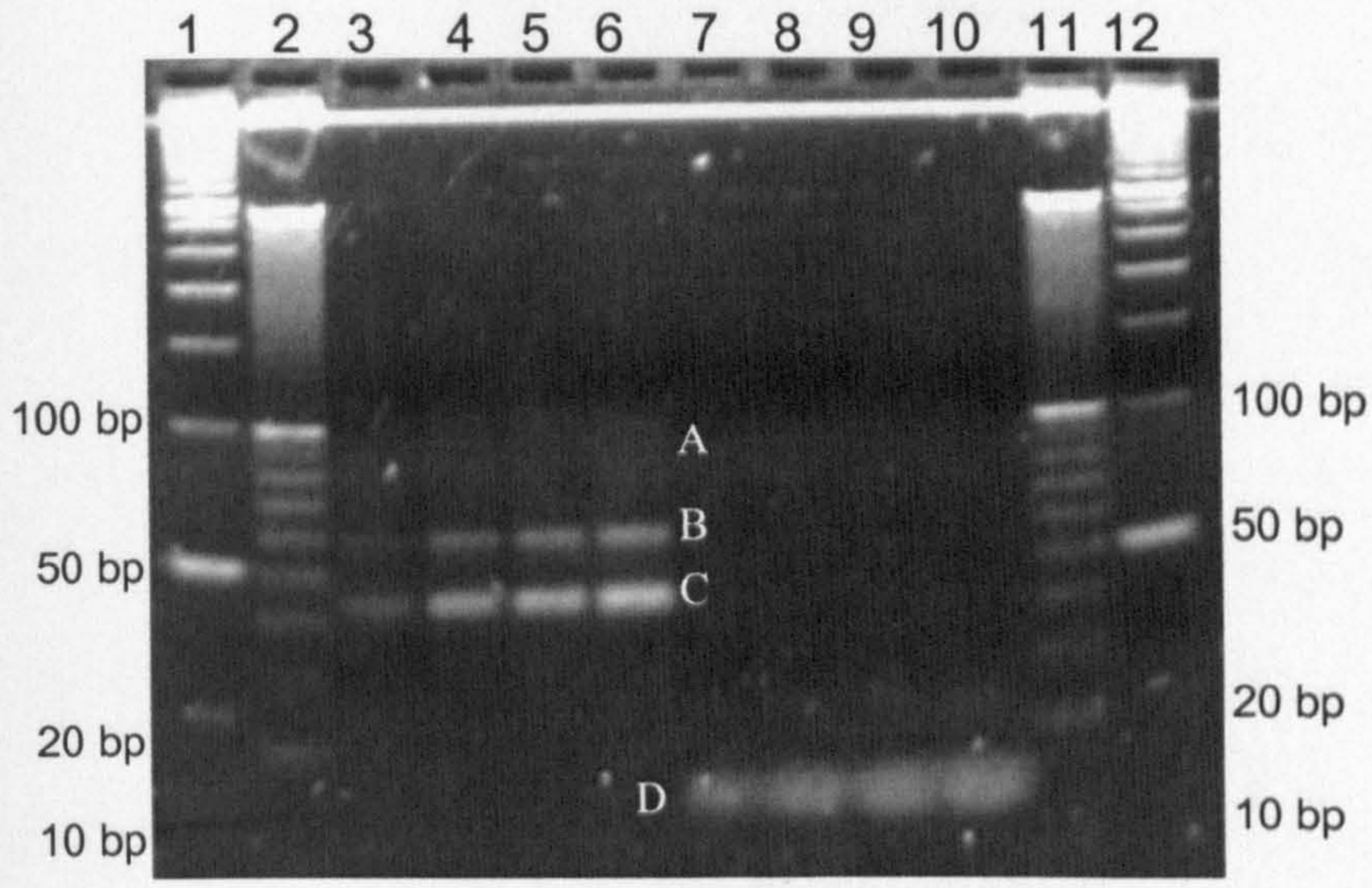


Figure 5.6 Lanes 3-6 show digested 96 bp (A) PCR product, partially digested 56 bp product (B) and fully digested 40 bp product (C) (1,2,3 and 4 μl from digestion product re-dissolved in 10 μl). Lanes 7-10 show double stranded INS3 standard (D) (1, 2, 3, 4 μg). Lanes 1, and 12 contain 25 bp DNA ladders, 2 and 11 contain 10 bp DNA ladders. (4% agarose, 70 V, 30 min).

A range of concentrations of Bpm1 was used to digest the PCR product. It was decided to use 20 Units per reaction as a compromise between cost and product yield. Figure 5.6 illustrates a gel where a digested sample is run alongside a double stranded deoxyoligonucleotide standard (14 bp). The PCR product is digested to form the 40 bp product but no small 16 bp product can be seen. Other gels showing more complete digestion did not yield the 16 bp product either (i.e. Figure 5.5.). There was a faint band in the appropriate position but this was hard to see under UV, and could not be detected using imaging apparatus (GeneQuant, Biorad, Hercules, Ca., USA).

The insert has been incorporated into the *supF* gene as shown by sequencing, and the PCR product can be digested to yield 40 basepair fragments, but the 16 basepair

fragment cannot be detected by electrophoresis. In theory this should not matter as we can look for specific sized ions at the mass spectrometry stage of the procedure. Work is still being carried out in our laboratory involving the detection of these 16 bp oligonucleotides.

5.3.1 Mass Spectrometry

Standard deoxyoligonucleotides were synthesised for all possible repair or mutagenic events along with their complementary strands; i.e. G(adduct)→ G (INS3Z/3Y), G(adduct)→ A (INS3ZA/3YT), G(adduct)→ C (INS3ZC/3YG) or G(adduct)→ T (INS3ZT/3YA). These standards were analysed by LC-MS. Figure 5.7 illustrates example HPLC chromatograms for INS3YA and INS3ZT. Standard deoxyoligonucleotide samples eluted in a single peak at around 7.5-8.0 minutes. Figure 5.8 shows examples of mass spectra for INS3YA and INS3ZT deoxyoligonucleotide standards. Figure 5.9 shows examples of deconvoluted mass spectra for INS3YA and INS3ZT deoxyoligonucleotide standards.

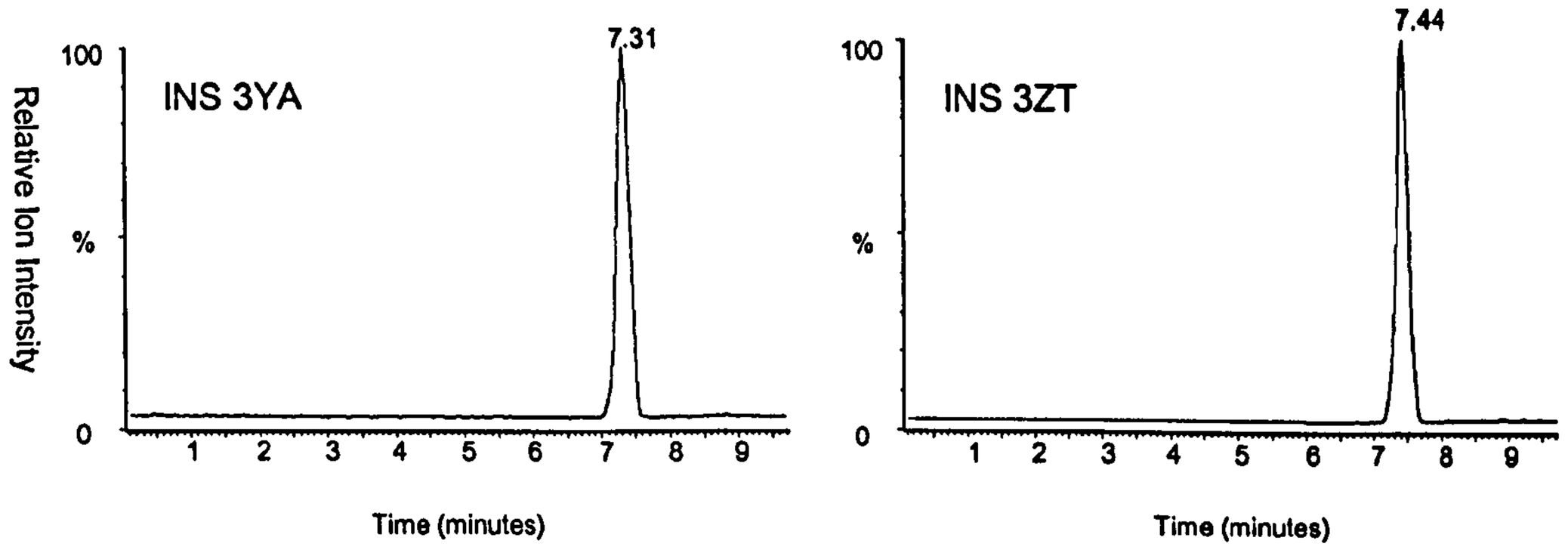


Figure 5.7 Illustration of HPLC chromatogram of INS3YA and INS3ZT standards.

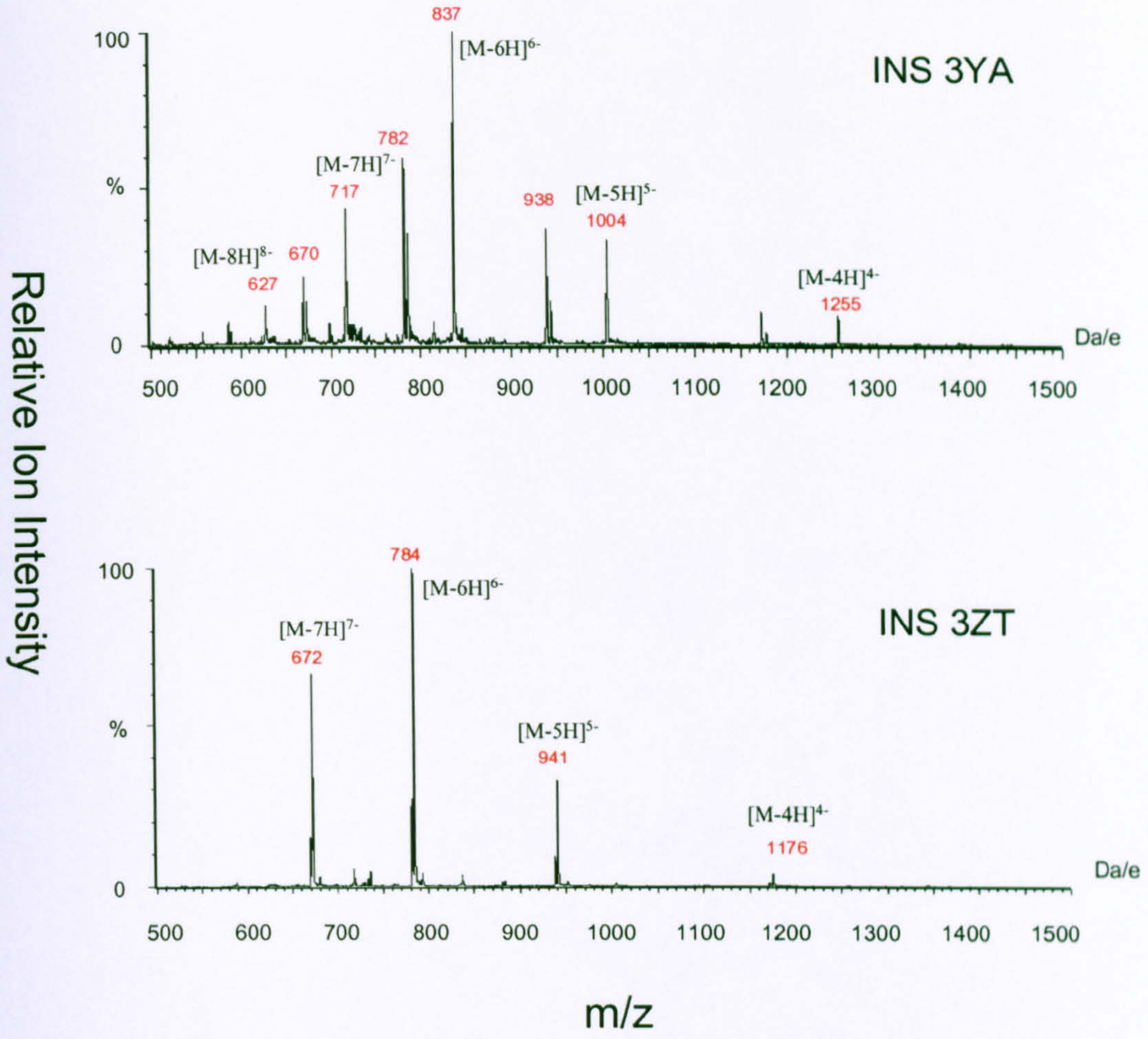


Figure 5.8 An example of mass spectra for INS3YA and INS3ZT standards.

Table 5.1 illustrates the observed masses for the standard deoxyoligonucleotides analysed. The difference in mass between the observed mass values and those calculated from the deoxyoligonucleotide sequences varied by a maximum of two mass units (0.04 %). All observed masses were higher than, or equal to, the expected masses rather than underestimates.

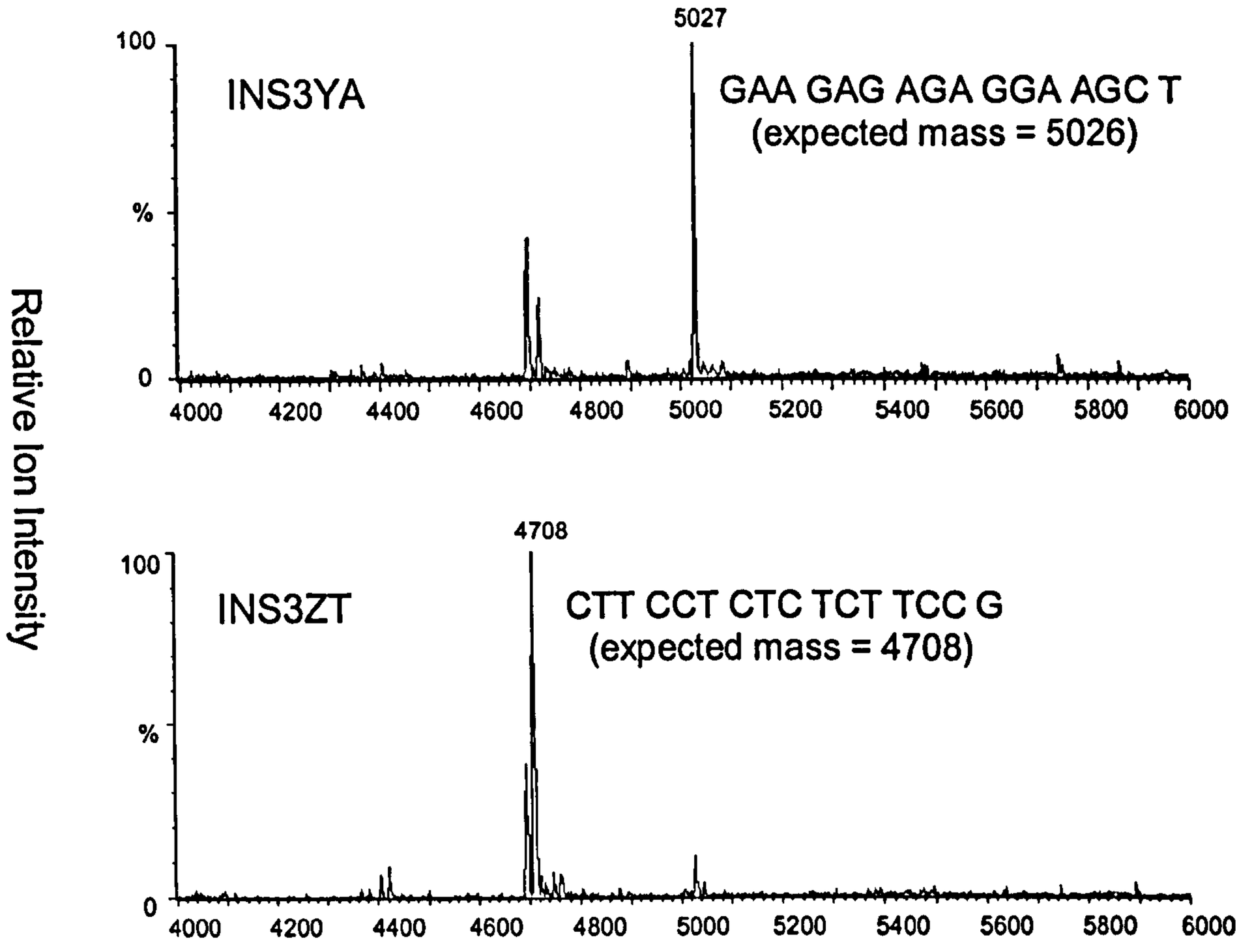


Figure 5.9 An example of deconvoluted to zero charge mass spectra showing the oligonucleotide masses for INS3YA and INS3ZT standards.

Table 5.1. Observed molecular ions for deoxyoligonucleotide standards

Standard	$[M-4H]^{4-}$	$[M-5H]^{5-}$	$[M-6H]^{6-}$	$[M-7H]^{7-}$	$[M-8H]^{8-}$	Observed Mass[b]	Expected Mass[c]	Difference (Obs-Exp)
3Y		999 (25)	833 (80)	714 (100)	626 (48)	**5003**	**5002**	1
3YA	1255 (3)[a]	1004 (22)	837 (70)	717 (100)	627 (10)	**5027**	**5026**	1
3YT	1253 (2)	1002 (20)	835 (65)	716 (100)	626 (25)	**5018**	**5016**	2
3YG		1007 (18)	839 (55)	719 (100)	629 (48)	**5043**	**5042**	1
3Z	1182 (20)	946 (100)	788 (85)	675 (25)		**4734**	**4733**	1
3ZA	1178 (25)	942 (100)	785 (75)	673 (30)	589 (10)	**4717**	**4717**	0
3ZT	1176 (5)	941 (30)	784 (100)	672 (65)		**4709**	**4708**	1
3ZC	1172 (10)	938 (80)	781 (100)	669 (10)	586 (2)	**4695**	**4693**	2

[a]Figures in brackets refer to arbitary ion intensities, compared to maximum (%)

[b]Deconvoluted to zero charge [c]Calculated from dNMP masses

Since single stranded standards could be detected and analysed successfully an annealed double stranded deoxyoligonucleotide standard was also analysed, as in the actual assay the digestion product will be double stranded. Figure 5.10 illustrates the HPLC chromatogram and mass spectra obtained for double stranded INS3. Double stranded deoxyoligonucleotides are denatured to single stranded deoxyoligonucleotides during the mass spectrometry analysis since the electrospray ionisation source temperature is 110°C.

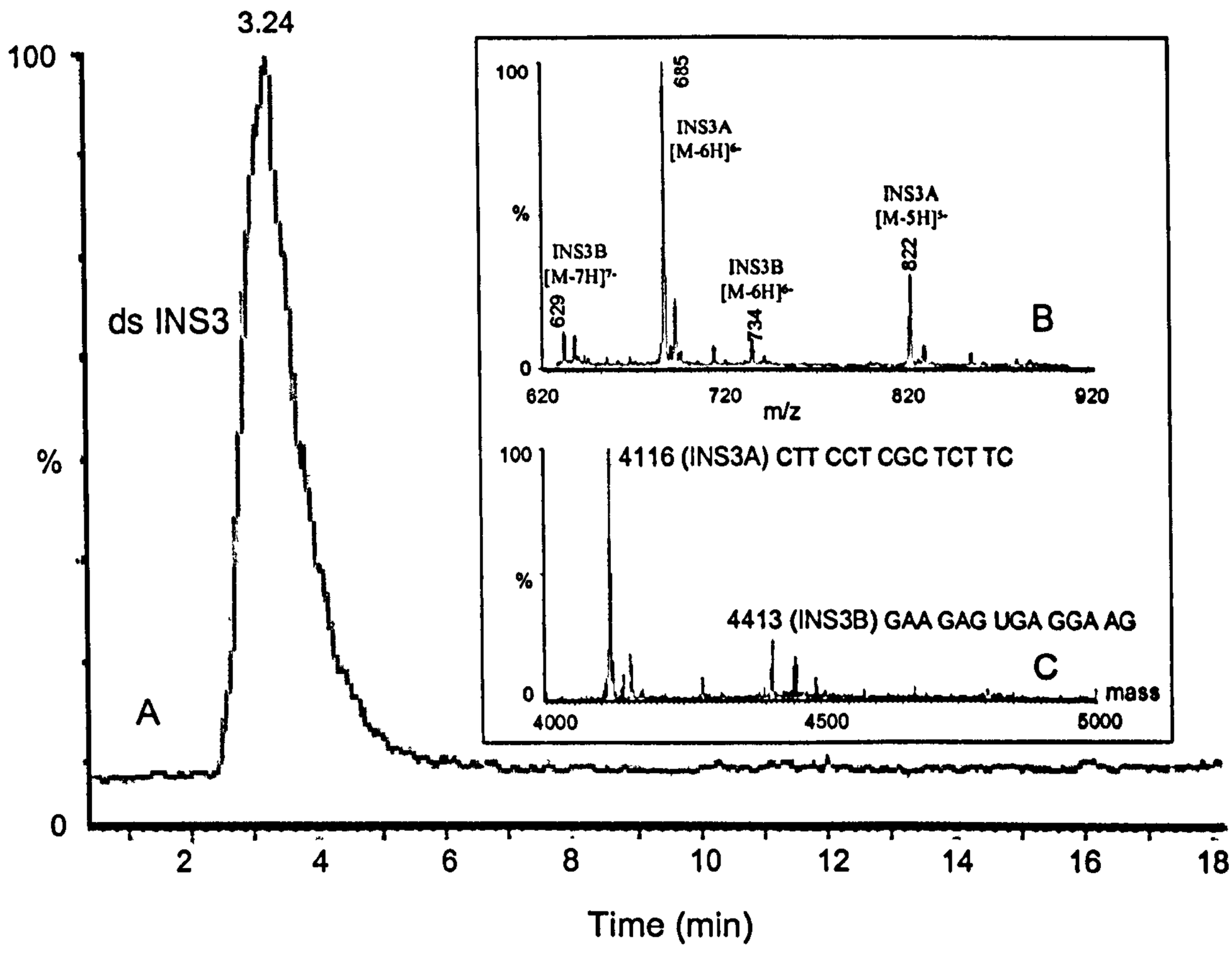


Figure 5.10 HPLC Chromatogram (A) showing double stranded insert INS3 and mass spectrum (B) and deconvoluted to zero charge mass spectrum (C) showing the resulting single stranded INS3A/B standards.

When deoxyoligonucleotide samples were recovered from plasmids and analysed in this way it was not possible to detect a mass corresponding to an expected 16-mer. Chromatography gave a peak of around the same elution time as the standards, indicating the presence of the required digestion product. The inability to detect the expected molecular ions by mass spectrometry may have been due to high salt concentrations in the sample therefore various methods were attempted to desalt the deoxyoligonucleotides. All precipitation steps were carried out with ammonium acetate rather than sodium acetate and different desalting procedures, Millipore concentrators, Biorad Bio-spin columns, Waters SPE desalting columns, were used along with C18 ziptips (Millipore). There was also a possibility that the small deoxyoligonucleotides were being trapped in the matrix of the enzyme removal columns which were used. Therefore, phenol/chloroform extraction was tried instead to remove the protein but this did not result in detectable products.

5.4 Discussion

The site selective mutagenesis assay described in this chapter offers considerable potential in future investigations of deoxyoligonucleotides containing important DNA lesions. There is the possibility of using any DNA sequence; be it genes, such as *p53*, which are already known to be important in the development of cancer, or genes which have yet to be identified, such as those important in the development of endometrial cancer in the case of tamoxifen. These preliminary experiments have shown that it is possible to insert a double stranded deoxyoligonucleotide into the *supF* gene of the pSP189 shuttle vector plasmid to knock out gene function. Replication of the shuttle vector plasmid can be achieved in SV40 transformed cell lines, of the appropriated lineage to the gene or compound being investigated, and mutations can then be induced by the DNA adducts, or repair can occur. Recovered plasmids which are screened in *E.coli* for the presence of inserts can be picked and sequenced to determine whether a mutation has been induced or repair has been achieved.

The development of the PCR part of the assay showed that the method of Laken *et al* (1998) could be used to amplify the deoxyoligonucleotides. Digestion of PCR products with Bpm1 was shown to yield the larger fragments from either side of the deoxyoligonucleotide of interest but, perhaps due to the electrophoresis visualisation system used, detection of the smaller fragments was not possible. Very faint bands were seen in the appropriate portion of the gels, but these were not detectable by the image analysis software used, only by eye using UV light. There are many reasons why these smaller fragments may not have been visible, for example; due to their

small size they may not bind sufficient ethidium bromide from the gel, or there may be a reduction in yield in the clean up steps, enzyme removal and desalting. Before this method is routinely used in this laboratory a method will have to be developed to prove that the 16 bp fragment is produced during digestion of the PCR product. This may involve the use of polyacrylamide gels, and end labelling of the oligonucleotides with ^{32}P.

Another problem encountered during mass spectrometric analysis was the high amounts of salt the sample had to come in contact with. All of the buffers used for the various enzymic steps contain sodium, potassium, magnesium and phosphate, all of which lead to suppression of the signal in the mass spectrometer. Ammonium acetate was exchanged for sodium acetate, and extra wash steps (using 70% and 100% ethanol) were added to the precipitation procedure but this did not improve mass spectral analysis. HPLC mobile phases, gradients and flow rates were also adjusted to try to limit the amount of salt entering the mass spectrometer. It is possible that the mass spectrometer used was more sensitive to salt concentration than that used by Laken *et al* (1998). It was shown that masses could be determined for the standard deoxyoligonucleotides to within 2 Daltons, although these would have been exposed to much lower levels of salt than samples recovered from cells. Some further development is needed to ensure that mass analysis can be achieved from recovered deoxyoligonucleotides before deoxyoligonucleotides with site selective DNA lesions can be used.

Any gene sequence can be investigated using a specific set of deoxyoligonucleotides. Since this method relies on chemical synthesis of the deoxyoligonucleotide inserts

any DNA lesions can be used, as long as they can be obtained in the form of phosphoramidites. If the DNA adduct phosphoramidites prove to be too unstable or cannot be synthesised then direct treatment of deoxyoligonucleotides with the appropriate mutagen can be utilised, although the adducted deoxyoligonucleotide will then need to be rigorously purified by HPLC and polyacrylamide gel electrophoresis.

One avenue which could be explored in the future could be the use of fluorescent hybridisation techniques to detect the mutations induced by the site selective DNA adducts. Anti-sense oligonucleotide probes could be made to detect the four possible outcomes of replication of the DNA adduct containing insert, i.e. G repaired to give G, or mutated to give C, A or T.

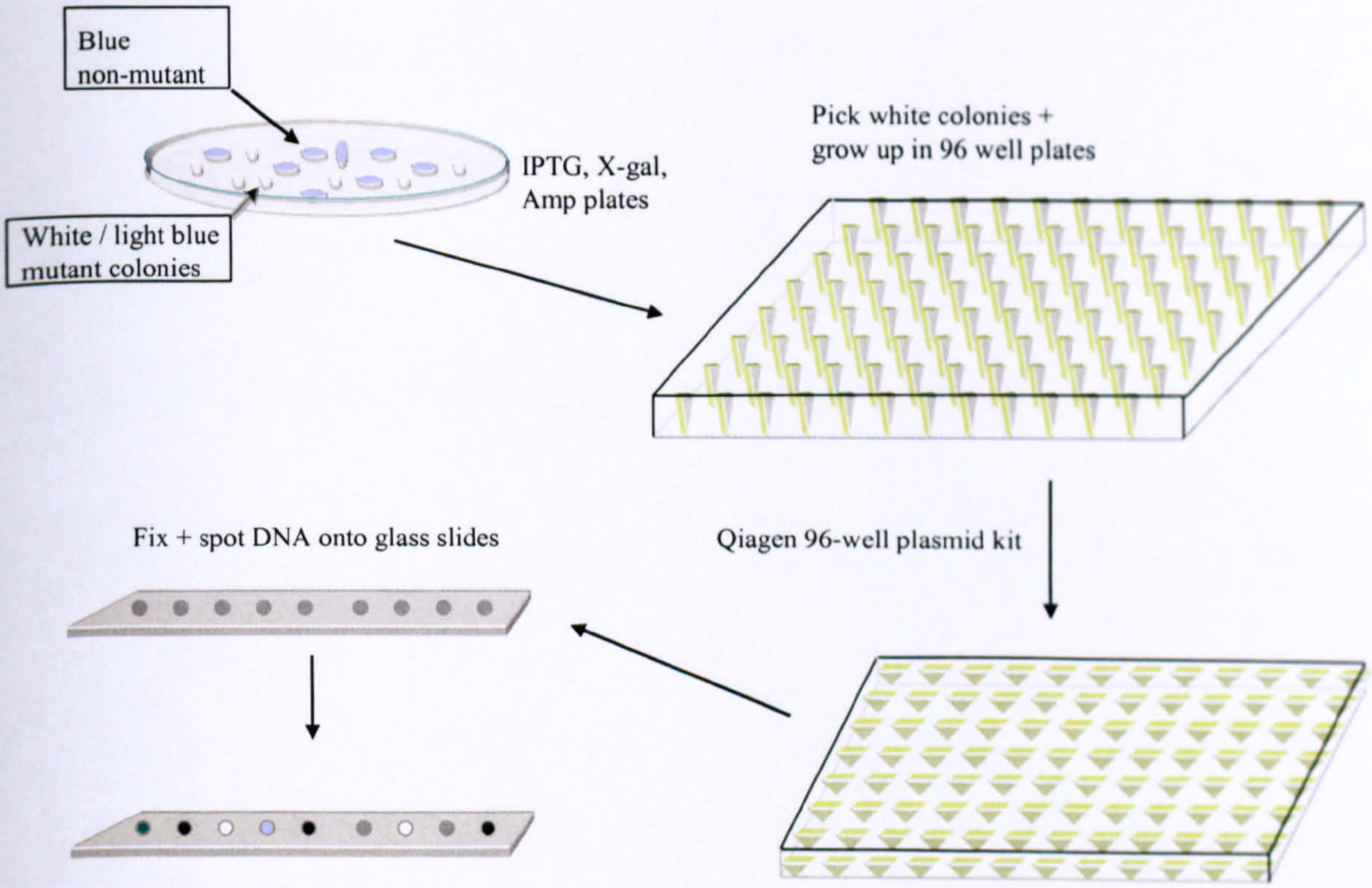


Figure 5.11 Site selective mutation assay using fluorescence hybridisation detection.

These could either have four differently coloured fluorescent probes, or the experiment could be carried out four times, i.e. for each possible sequence, using a single coloured probe. The identity of the base in question could then be deduced from the colour detected. This method may have the added benefit that the plasmid preparation could be automated using apparatus available at the MRC Toxicology Unit at the time, as could the picking of colonies and spotting of plasmid DNA onto slides. The mass spectrometry mutation detection method could also become partially automated, with the use of HPLC auto samplers and by using 96 well PCR plates.

The development of a fluorescent mutation detection technique is currently being investigated in our laboratory. This method will be utilised in parallel with the mass spectrometry technique to allow for the most sensitive and accurate detection of site selective mutations induced by genotoxic chemicals.

5.5 Summary

The potential advantages of the proposed assay are:

1. The adduct is being replicated in human cells and is present in double stranded DNA.
2. Any DNA sequence can be used enabling the adduct to be placed in various sequence contexts so that the effects of neighbouring bases on adduct mutagenicity can be examined. In addition, the adduct can be incorporated into a variety of important genes.

3. The mass spectrometry or fluorescence based detection methods will allow for high-throughput, rapid analysis of mutant sequences.
4. Once information is gained on the types of mutations a particular adduct induces in a known gene then these detection methods could potentially be used to screen human populations exposed to the chemical under investigation for the presence of this gene mutation (Laken, *et al.*, 1998).

Chapter 6

Conclusions and Summary

The aim of the work described in this thesis was to establish and utilise mutagenesis assays for investigating the mutagenic effects of carcinogenic agents. Initially this required validation of the *supF* assay in our laboratory by using UVC radiation to introduce damage into the pSP189 plasmid shuttle vector. The *supF* assay was then used to examine the mutagenicity of reactive metabolites of tamoxifen, α-acetoxytamoxifen and 4-hydroxytamoxifen quinone methide (4-OHtamQM) in human Ad293 cells. Repair proficient and deficient cells were also used to investigate the effect of DNA repair on α-acetoxytamoxifen induced DNA damage. The mutagenicity of BPDE and UV radiation was investigated using binary treatments. Finally, as an extension of this work a site-selective mutation assay was developed using the *supF* assay and mass spectrometric analysis to detect mutations in synthetic oligonucleotides.

Validation of the *supF* forward mutation assay was successfully achieved in our laboratory using UV radiation. Although the results presented here are not entirely consistent with those reported previously, Ad293 cells have not been used before to investigate UV mutagenesis. A relationship between UV dose and mutation frequency was shown, along with the fact that the GC→AT transition was the most prevalent mutation. This does agree with data from published studies analysing UV irradiated *supF* containing plasmid replicated in XP cells. The assay was shown to be suitable for use in this laboratory for future mutagenesis studies on a variety of pharmaceutical and environmental mutagens.

BPDE treatment and UVB or UVC irradiation all induced the expected mutations, i.e. GC→TA transversions for BPDE and GC→AT transitions for UV. Interestingly,

combined treatments induced a mixture of these mutations, although in the case of BPDE combined with UVB, it seems that the UVB radiation is the stronger mutagen as GC→AT is the preferred mutation, and sites of mutation hotspots are shared with the UVB only mutation spectrum. When the treatment order was reversed (BPDE then UVC compared to UVC then BPDE) there does not appear to be much change in the types of mutations induced, but there is a difference in the mutation distribution and frequency in the *supF* gene. It would appear that irradiation of BPDE adducted plasmid results in some chemical activation of the adducts or the production of reactive radical species which increases mutation frequency. This increase is greater than when UV irradiated plasmid is treated with BPDE. This phenomenon warrants further investigation.

It has been shown that the minor tamoxifen DNA adduct, α-(deoxyguanosin-N^2-yl)-4-hydroxytamoxifen, formed by 4-OHtamQM is more mutagenic than the major tamoxifen DNA adduct, α-(deoxyguanosin-N^2-yl)-tamoxifen, formed by α-acetoxytamoxifen. It was also shown that these two reactive metabolites of tamoxifen induced different types and distributions of mutations in the *supF* gene. Investigating the effect of DNA repair on tamoxifen DNA adducts showed that there was a difference in mutation distribution between XP cells and their normal counterparts but there was no real increase in mutation frequency when nucleotide excision repair was lacking.

Development of a site-selective mutation assay using mass spectrometry looks promising. It has been demonstrated that an oligonucleotide can be inserted into the *supF* gene to act as a frameshift mutation. This results in the growth of mutant

colonies containing the oligonucleotide insert. Recovery of this insert followed by analysis by mass spectrometry will allow us to investigate the mutagenicity of various DNA adducts and sequence contexts. This method, however, does need a small amount of development in this laboratory before it can be used routinely, but will complement the standard *supF* assay and provide further mechanistic information aimed at elucidating mutagenic processes.

Chapter 7

Appendices and References

Appendices

Appendix 1. Raw ^{32}P-postlabelling data from BPDE / UV treatment of pSP189 plasmid (Part B)

	Value 1	Value 2	Value 3	Average	S.D.	Standard Error
Solvent control	0	0.0032	0.001	0.0014	0.0016	0.00095
BPDE	1.03	0.4338	0.15	0.54	0.45	0.26
UVB		0	0.00002	0.00001	1.41E-05	0.00001
UVC		0.0029	0.0005	0.0017	0.0017	0.0012
UVB then BPDE	2.95	1.5798	0.332	1.62	1.31	0.76
UVC then BPDE	4.61	0.9362	0.57	2.04	2.23	1.29
BPDE then UVB	3.81	1.4090	0.82	2.01	1.58	0.91
BPDE then UVC	10.85	2.0156	0.5	4.46	5.59	3.23

Adducts per 10^5 nucleotides

Appendix 2. Raw ^{32}P-postlabelling data from α-acetoxytamoxifen treatment of pSP189 plasmid

	Value 1	Value 2	Value 3	Value 4	Average	S.D.	Standard Error
Solvent Control	0	0	0	0	0	0	0
10 μM	5791.21	3947	5609.14	4542.59	4972	877.89	438.94
25 μM	16331.6	14989.6	15564.5	16790.5	15919	799.85	399.93
50 μM	21848.6	24854.1	29879.1	17435.5	23504	5229.15	2614.58

Adducts per 10^8 nucleotides

Appendix 3. Raw ^{32}P-postlabelling data from 4-OHtamQM treatment of pSP189 plasmid

	Value 1	Value 2	Average	S.D.	Standard Error
Solvent Control	0	0	0	0	0
50 μM	553.08	464.85	509	62.39	31.19
100 μM	960.72	630.92	796	233.20	116.60
250 μM	1885.33	2022.16	1954	96.75	48.38

Adducts per 10^8 nucleotides

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DNA Adducts Formed from 4-Hydroxytamoxifen Are More Mutagenic than Those Formed by α-Acetoxytamoxifen in a Shuttle Vector Target Gene Replicated in Human Ad293 Cells†

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ABSTRACT: The drug tamoxifen, used to treat breast cancer, causes liver cancer in rats and endometrial cancer in women. Tamoxifen forms liver DNA adducts in both short- and long-term dosing of rodents, and DNA adducts have also been reported in tissues of women undergoing tamoxifen therapy. It is not known if the induction of endometrial cancer in women is through these DNA adducts or through the estrogenic nature of the drug. In this study, we have investigated the mutagenicity of two model reactive intermediates of tamoxifen, α-acetoxytamoxifen and 4-hydroxytamoxifen quinone methide (4-OHtamQM). These form the same DNA adducts as those found in tamoxifen-treated rats. The two compounds were used to treat the pSP189 plasmid containing the *supF* gene, which was replicated in Ad293 cells before being screened in indicator bacteria. Plasmid reacted with 4-OHtamQM was more likely to be mutated (2−7-fold increase) than that reacted with α-acetoxytamoxifen, despite having a lower level of DNA damage (12−20-fold less), as assayed by ^{32}P-postlabeling. The two compounds induced statistically different mutation spectra in the *supF* gene. The majority of mutations in α-acetoxytamoxifen-treated plasmid were GC →TA transversions while GC→AT transitions were formed in 4-OHtamQM-treated plasmid. 4-OHTamQM-treated DNA induced a larger proportion of multiple mutations and large deletions compared to α-acetoxytamoxifen. Sites of mutational hotspots were observed for both compounds. In conclusion, the quantitatively minor DNA adduct of tamoxifen (dG-N^2-4-hydroxytamoxifen) is more mutagenic than the major tamoxifen DNA adduct (dG-N^2-tamoxifen).

Tamoxifen [*trans*-(*Z*)-1-[4-[2-(dimethylamino)ethoxy]phenyl]-1,2-diphenyl-1-butene] is an antiestrogenic drug used in the adjuvant therapy of breast cancer. It has recently been approved by the United States Food and Drug Administration (FDA) for use as a chemopreventative agent in women at high risk of breast cancer. This was based on the finding that tamoxifen achieved a statistically significant 49% reduction in the incidence of invasive breast cancer in women with increased risk of the disease (*1*). However, both chemopreventative and therapeutic dosing strategies have been shown to slightly increase the risk of endometrial cancer in comparison to controls (*2, 3*). In addition, both short-term and long-term treatment of rats with tamoxifen results in the induction of hepatocellular carcinomas preceded by the formation of large amounts of hepatic DNA adducts (*4*−*6*). Tamoxifen DNA adducts have also been reported in leukocytes and endometrial tissue of women undergoing tamoxifen therapy (*7, 8*), adding to concerns over the long-term health hazards of tamoxifen.

In rat liver, DNA damage is initiated only after metabolic activation of tamoxifen (*9*). The principal metabolic pathway in vivo involves α-hydroxylation (*10*−*13*) followed by hydroxysteroid sulfotransferase mediated sulfate conjugation (*14, 15*). Loss of this sulfate moiety leaves a carbocation (*16*), promoting nucleophilic attack by the N^2-amino group of deoxyguanosine to form α-(deoxyguanosin-N^2-yl)tamoxifen (dG-N^2-tam)[1] (*17, 18*) (Figure 1). This adduct exists as four diastereoisomers, with the trans-forms accounting for one of the major adducts in rat liver DNA. While α-hydroxytamoxifen can react directly with DNA, the synthetic *O*-sulfonate gives greater than a 180-fold increase in adduct yield. However, the α-sulfonate of tamoxifen is very short-lived, so a model ester, α-acetoxytamoxifen, is often used for studies in vitro. Incubation of DNA with α-acetoxytamoxifen in vitro produces the same dG-N^2-tamoxifen adducts formed in rats and humans after treatment with tamoxifen (*19, 20*).

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[1] Abbreviations: 4-OHtamQM, 4-hydroxytamoxifen quinone methide; dG-N^2-4-hydroxytamoxifen, α-(N^2-deoxyguanosinyl)-4-hydroxytamoxifen; dG-N^2-tamoxifen, α-(N^2-deoxyguanosinyl)tamoxifen.

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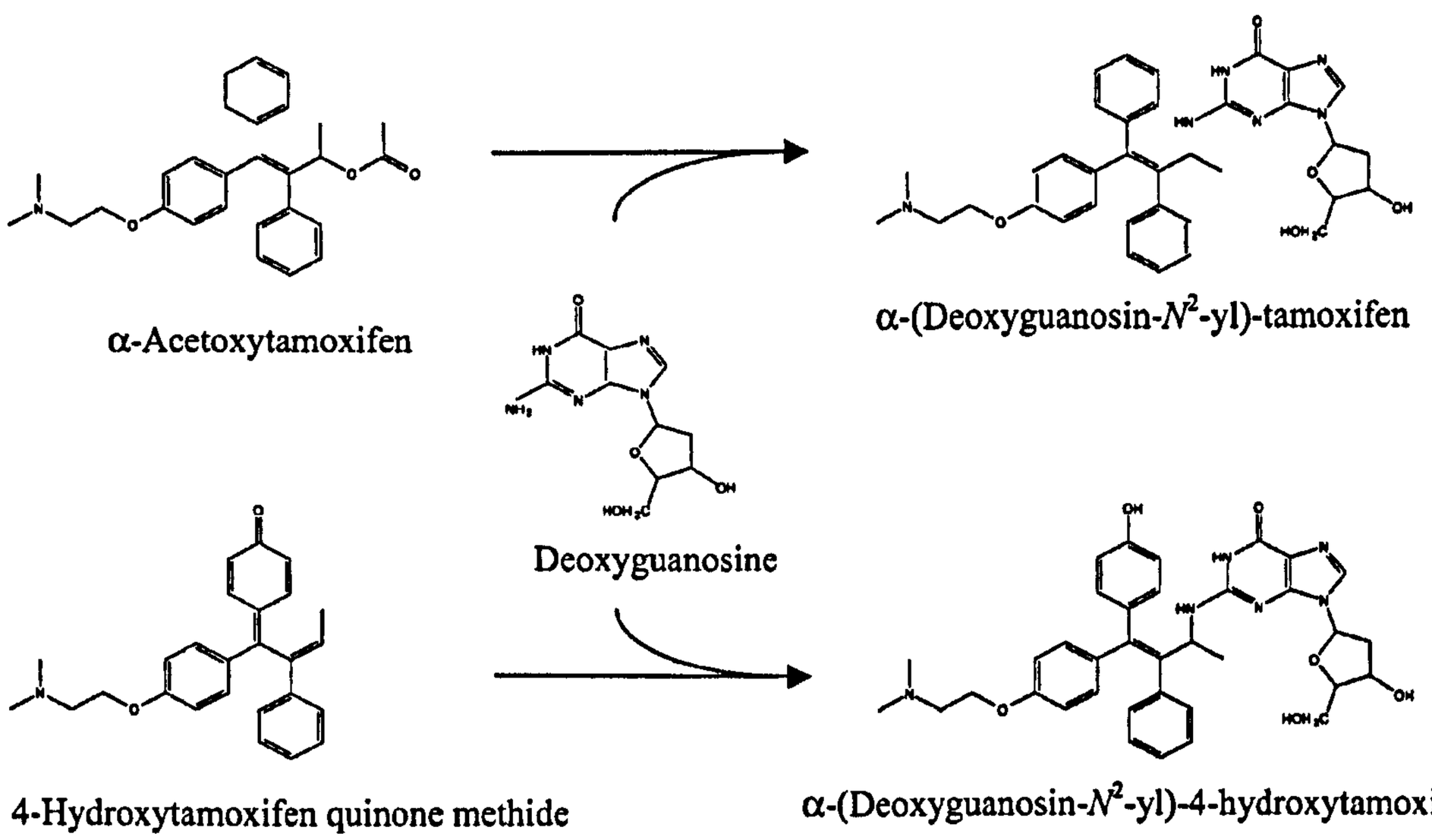

FIGURE 1: Reaction of α-acetoxytamoxifen with deoxyguanosine yields the major α-(deoxyguanosin-N^2-yl)tamoxifen adduct in rats. Reaction of 4-hydroxytamoxifen quinone methide with deoxyguanosine yields a minor adduct, α-(deoxyguanosin-N^2-yl)-4-hydroxytamoxifen, in rats.

A second major tamoxifen metabolite, 4-hydroxytamoxifen (*21*), is also thought to be activated to a DNA reactive species either via α-hydroxylation, presumably followed by α-sulfation, or possibly via formation of a quinone methide (*22*). Studies in rats have reported the formation of DNA adducts derived from 4-hydroxytamoxifen (*23*) although in rat liver these have been detected at much lower levels than those derived from α-hydroxytamoxifen (*24*).

Recent studies have shown that site-specific dG-N^2-tamoxifen adducts induce primarily GC→TA transversions in COS-7 cells (*25*). In addition, administration of tamoxifen causes GC→TA transversions in both the *cII* and *lacI* genes in lambda/*lacI* transgenic rats (*26, 27*). Although most efforts to date have focused on the major dG-N^2-tamoxifen adducts, we have previously reported that adducts formed by 4-hydroxytamoxifen are 2 orders of magnitude more mutagenic in *E. coli* than those arising from α-acetoxytamoxifen (*28*).

To compare the mutagenicity of tamoxifen DNA adducts replicated in human cells, we have used the *supF* forward mutation assay to study mutations induced by α-acetoxytamoxifen and 4-hydroxytamoxifen quinone methide (4-OH-tamQM). The *supF* assay (*29, 30*) has been widely used to study the mutagenicity of compounds that form bulky adducts when reacted with DNA. The assay detects 97% of possible base substitutions within the 85 base pair *supF* gene (*31*) as well as deletions and insertions, and because the plasmid is treated in vitro, aliquots of the treated DNA can be analyzed for adduct quantification in parallel to the mutation assay. To this end, a previously developed ^{32}P-postlabeling method for analysis of tamoxifen DNA adducts was used (*24*). The results we report here show that minor tamoxifen DNA adducts may contribute significantly to the mutagenicity of the compound in mammalian cells.

MATERIALS AND METHODS

Materials. The trans isomer of α-hydroxytamoxifen was synthesized using the method described by Foster and coworkers (*32*). *trans*-α-Acetoxytamoxifen was prepared from *trans*-α-hydroxytamoxifen using the published method (*17*). [γ-^{32}P]ATP (>185 TBq/mmol, >5000 Ci/mmol, 370 MBq/mL) was purchased from Amersham, Buckinghamshire, U.K. T4 polynucleotide kinase (3′-phosphatase free) and calf spleen phosphodiesterase were bought from Roche, Lewes, East Sussex, U.K. All other chemicals were from Sigma, Poole, Dorset, U.K., unless otherwise stated.

Shuttle Vector Plasmid, Bacterial Strain, and Cell Lines. The plasmid pSP189 containing the *supF* gene (*30*) and *E. coli* strain MBM7070 were gifts from M. Seidman, Oncor Pharmaceuticals, Gaithersburg, MD. Human embryonic adenovirus-transformed kidney cells (Ad293) were cultured from cells previously provided by Dr. A. Dipple, National Cancer Institute, Frederick, MD. Ad293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Life Technologies Ltd., Paisley, U.K.) at 37 °C in 5% CO_2 in air.

Treatment of DNA with α-Acetoxytamoxifen. Aliquots of pSP189 plasmid (200 μg, in 200 μL of Tris−EDTA buffer, pH 8.0) were incubated with 10, 25, and 50 μM α-acetoxytamoxifen in ethanol at 37 °C for 18 h. A control DNA incubation, to which only ethanol was added, was also carried out. The samples were extracted with 3 × 400 μL of water-saturated ethyl acetate to remove unreacted α-acetoxytamoxifen. Plasmid DNA was then precipitated with 3 M sodium acetate/ice-cold ethanol and redissolved in 200 μL of sterile tissue culture grade water.

Treatment of DNA with 4-Hydroxytamoxifen Quinone Methide. 4-Hydroxytamoxifen (6.52 mg) was activated to a quinone methide with silver(II) oxide (42.92 mg in 1.8 mL of dry chloroform, stirred for 30 min) using established procedures (*33*). The reaction mixture was filtered and dried to a yellow−brown residue under nitrogen. This was dissolved in ethanol/acetonitrile (1:1 v/v), and added to 100 μg of pSP189 plasmid. Final concentrations of 0, 50, 100, and 250 μM were used. After incubation at 37 °C for 18 h,

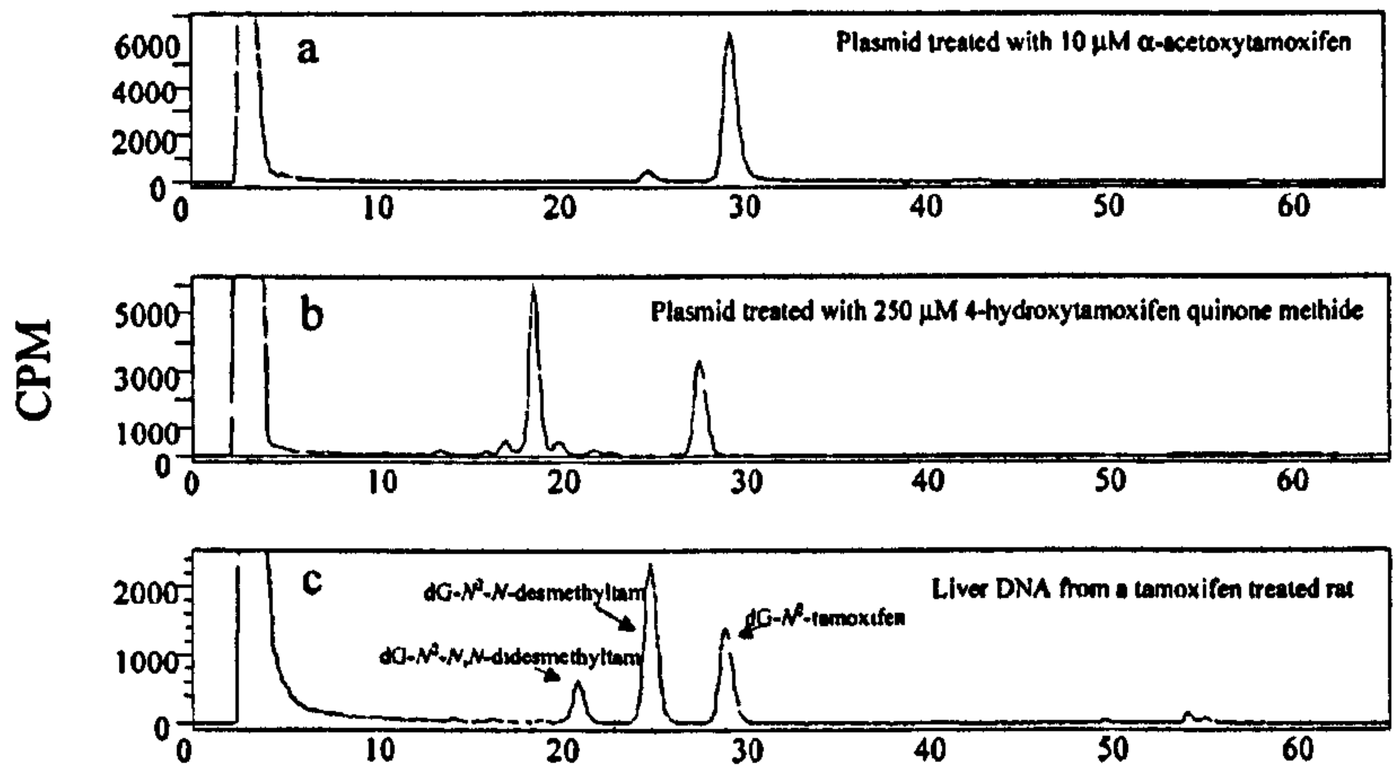

FIGURE 2: Representative radioactive HPLC chromatograms obtained for plasmid treated with α-acetoxytamoxifen (a), 4-hydroxytamoxifen quinone methide (b), and liver DNA from a tamoxifen-treated Wister Han Rat (40 mg/kg tamoxifen in diet for 6 months)(c).

the unreacted 4-hydroxytamoxifen quinone methide was extracted from the plasmid with diethyl ether (5 × 400 μL). Plasmid DNA was precipitated with 3 M sodium acetate/ice-cold ethanol and redissolved in 200 μL of sterile tissue culture grade water.

^{32}P-Postlabeling of α-Acetoxytamoxifen or 4-Hydroxytamoxifen Quinone Methide-Treated Plasmid. To quantify the number of tamoxifen DNA adducts on the treated plasmids, aliquots of plasmid DNA (5 μg) were analyzed by the ^{32}P-postlabeling assay, incorporating a nuclease P1 enhancement step. ^{32}P-Postlabeled nucleotides were separated by HPLC and measured by on-line radiochemical detection (*24*).

Transfection and Transformation. Subconfluent cells were transfected with α-acetoxytamoxifen- or 4-OHtamQM-treated plasmid (10 μg per 9 cm culture plate) using the calcium phosphate precipitation technique (*34*). After 48 h, plasmid was recovered using plasmid purification kits (Qiagen, Crawley, West Sussex). Aliquots of recovered plasmid were used to transform electrocompetent MBM7070 *E. coli* by electroporation using Gene Pulser apparatus (Biorad, Hercules, CA). Transformants were plated onto LB agar plates containing ampicillin (100 μg/mL), 5-bromo-4-chloro-3-indolyl-β-D-galactose (X-gal) (75 μg/mL), and isopropyl-β-D-thiogalactoside (IPTG) (25 μg/mL). Mutant colonies were white when grown on X-gal-containing media, whereas wild-type colonies were blue.

Sequencing. Plasmid was extracted from white mutant colonies using plasmid purification kits (Qiagen, Crawley, West Sussex) and sequenced using the primer 5′-GGCGA-CACGGAAATGTTGAA-3′ (Protein and Nucleic Acid Chemistry Laboratory, Hodgkin Building, University of Leicester, U.K.). The pSP189 shuttle vector contains an 8 base 'signature sequence' giving 4^8 (65 536) possible unique sequences (*30, 31*). Any mutants with a duplicated 'signature' were excluded from further analysis. Poisson distribution analysis was used to assess the randomness of spectra. Hotspots were assumed when the number of mutations observed was 4-fold or more greater than the number expected for a random (Poisson) distribution.

RESULTS

^{32}P-Postlabeling Analysis of Plasmid pSP189 Treated with α-Acetoxytamoxifen or 4-Hydroxytamoxifen Quinone Methide. To assess the mutagenic effects of tamoxifen reactive intermediates derived from α-hydroxytamoxifen and 4-hydroxytamoxifen, the shuttle vector plasmid pSP189 was modified by in vitro reaction with α-acetoxytamoxifen and 4-OHtamQM, respectively. ^{32}P-Postlabeling analysis of the modified plasmids revealed HPLC adduct profiles similar to previous reports for each compound as illustrated in Figure 2 (*24, 28*). As judged by HPLC, treatment with α-acetoxytamoxifen results in the formation of the dG-N^2-tamoxifen DNA adduct that has previously been detected in liver tissue of tamoxifen-dosed rats (Figure 2a). An additional minor peak is also observed eluting just prior to the major adduct, which, based on retention time, is the N-demethylated dG-N^2-tamoxifen adduct (Figure 2c) (*35*). Following incubation of 4-OHtamQM with plasmid DNA, two main ^{32}P-postlabeled adduct peaks are detected (Figure 2b). Although these peaks do not coelute with any adduct peaks detected in DNA from rat liver tissue shown in Figure 2c, we have previously demonstrated the presence of up to 12 adduct peaks in similarly treated rats, and 1 of these coelutes with the main 4-hydroxytamoxifen-derived adduct (*24*). The major products of the reaction of 4-OHtamQM, produced by chemical oxidation, with DNA are known to be isomers of a 4-hydroxylated form of dG-N^2-tam (*22*). This reactive intermediate can also be generated enzymatically. We have previously shown that incubation of 4-hydroxytamoxifen with horseradish peroxidase yields one major ^{32}P-postlabeled adduct peak, which corresponds to the larger of the two adduct peaks observed in this study (*24, 28*). This difference in adduct profile is probably due to the different methods used to activate 4-hydroxytamoxifen. The additional adduct, which is present at low levels with peroxidase activation, may be an isomer of dG-N^2-4-hydroxytam or may be an as yet unidentified adduct. Quantification of adduct levels over the concentration range used demonstrated a dose-dependent increase in DNA damage for each compound (Table 1). Higher concentrations of 4-OHtamQM were used in the incubations compared to α-acetoxytamoxifen, as the former is known to generate lower levels of adducts (*28*). In the present study, 4-OHtamQM (50 μM) induced a 50-fold lower level of DNA adducts than an equimolar dose of α-acetoxytamoxifen. The degree of pSP189 modification by α-acetoxytamoxifen equates to a level of 0.5, 1.6, or 2.5

Table 1: Mutation Frequency and Adduct Number Induced by α-Acetoxytamoxifen and 4-Hydroxytamoxifen

treatment	mutation frequency[a]	adduct number[b] (±SD)
	α-Acetoxytamoxifen	
control[c]	6.6	0 ± 0
10 μM	10.5	50 ± 8.8
25 μM	9.3	160 ± 8
50 μM	32.3	240 ± 52
	4-Hydroxytamoxifen	
control[c]	3.3	0 ± 0
50 μM	6.2	5 ± 0.6
100 μM	64.4	8 ± 2.3
250 μM	72.1	20 ± 1

[a] Mutation frequency per 10^4 colonies. [b] Adduct number per 10^6 nucleotides. [c] Mutation frequency of plasmid dissolved in water only was 0.2×10^{-4}.

Table 2: Types of Sequence Alterations in the *supF* Gene of pSP189 Plasmids Treated with α-Acetoxytamoxifen and 4-Hydroxytamoxifen Quinone Methide

	number of plasmids with mutations (%)	
types	α-acetoxytam	4-hydroxytam
base substitutions	132 (90)	96 (62)
single	109 (74)	49 (32)
tandem	3 (2)	1 (0.6)
multiple	20 (14)	46 (30)
frameshifts	15 (10)	58 (38)
single base deletion	3 (2)	2 (1.3)
>2 base deletion	12 (8.2)	50 (32)
single base insertion	0 (0)	3 (1.9)
>2 base insertion	0 (0)	3 (1.9)
total plasmids sequenced	147 (100)	154 (100)

adducts per plasmid (4952 base pairs) for the 10, 25, and 50 μM doses, respectively. The three treatments with 4-OHtamQM (50, 100, and 250 μM) induced approximately 0.05, 0.1, and 0.2 adducts per plasmid.

Mutation Frequency in the supF Gene. While the spontaneous mutation frequency of plasmid suspended in water (2 in 10^5) was similar to data previously published (*36*), the solvent-treated control plasmid did show increased mutation frequency in the absence of α-acetoxytamoxifen or 4-OHtamQM (Table 1). Table 1 also shows that the total mutation frequency increased with dose, and hence adduct level, after treatment with both α-acetoxytamoxifen and 4-OHtamQM. The 4-OHtamQM induced a 2−7-fold greater increase in mutation frequency than treatment with α-acetoxytamoxifen, even though the number of adducts produced by 4-OHtamQM treatment was 10−20-fold lower than by α-acetoxytamoxifen treatment.

Mutation Types Found in the supF Gene. White mutant colonies were collected, and the *supF* gene was sequenced to identify the types of mutation and their location within the gene. The majority of mutations induced by both α-acetoxytamoxifen and 4-OHtamQM were base substitutions (90% and 62% of all mutants, respectively) as shown in Table 2. In α-acetoxytamoxifen-treated cells, most of these were in the form of single base substitutions (74%), with a few tandem (2%) and a larger proportion of multiple substitutions (14%, 2 or more substitutions at nonadjacent sites along the *supF* gene). In 4-OHtamQM-treated cells, there was an almost equal amount of single and multiple substitutions (30% and 32%, respectively) along with a single tandem substitution (0.6%). Frameshift mutations were relatively infrequent in α-acetoxytamoxifen-treated cells (10%) but more abundant in 4-OHtamQM-dosed cells (38%). Of these frameshifts, insertions were uncommon for both treatments (0−3.8%). Deletions, especially in the form of large deletions (greater than two adjacent bases deleted), were more common. Treatment with α-acetoxytamoxifen induced 2% single base deletions and 8.2% large (greater than 2 bases) deletions. Treatment with 4-OHtamQM induced 1.3% single base deletions and a high number (32%) of large (greater than 2 bases) deletions.

Figure 3 illustrates the effect of increasing dose on the mutation profiles induced by α-acetoxytamoxifen and 4-OHtamQM. Each type of substitution is expressed as a percentage of the total substitutions detected for each dose and corrected relative to the mutation frequency observed at the highest dose for each treatment. For α-acetoxytamoxifen-modified plasmid, as the dose increases so does the proportion of transversions compared to transitions. Overall, the most common single base substitutions are GC→TA transversions, occurring over 2-fold more frequently than GC→AT transitions (Figure 3). The mutation pattern for this compound is consistent with the fact that α-acetoxytamoxifen binds predominantly to deoxyguanosine; therefore, most substitutions would be expected to occur at GC base pairs. In α-acetoxytamoxifen-treated plasmids with multiple mutations in the *supF* gene, the predominant substitutions are GC→AT transitions, followed by GC→TA transversions. In the single base substitutions induced by 4-OHtamQM treatment, most of the mutations are at GC base pairs, with the major substitution, GC→AT transitions, up to 2-fold more prevalent than GC→TA transversions. For multiple mutations, the number of GC→TA transversions slightly exceeds the number of GC→AT transitions.

Mutation Spectra in the supF Gene. The distribution of base substitution mutations within the *supF* gene for plasmids dosed with α-acetoxytamoxifen and 4-OHtamQM is shown in Figure 4. Multiple base substitutions, which are illustrated on separate spectra, were included due to the large proportion induced by 4-OHtamQM; almost one-third of all mutants had multiple mutations. When these spectra are compared using the Hyperg program (*37*), they are all found to be significantly different from each other. There are six hotspots in the spectrum of single base substitutions induced by α-acetoxytamoxifen. A hotspot is defined as a site where the number of mutations observed was 4-fold or more greater than the number expected for a random Poisson distribution. These are at positions 105, 118, 122, 159, 160, and 163, all of which are at GC sites. In the spectrum of multiple α-acetoxytamoxifen substitutions, there are two definite hotspots at positions 156 and 168 and two more 'borderline' hotspots at positions 155 and 174, all of which are at GC sites. Four hotspots are apparent in the spectrum of single base substitutions induced by 4-OHtamQM at positions 129, 139, 155, and 156. The multiple base substitution spectrum has four hotspots at positions 100, 133, 156, and 174. All of these hotspots are also at sites of GC base pairs. Positions 155, 156, and 174 had hotspots in two or more of the spectra.

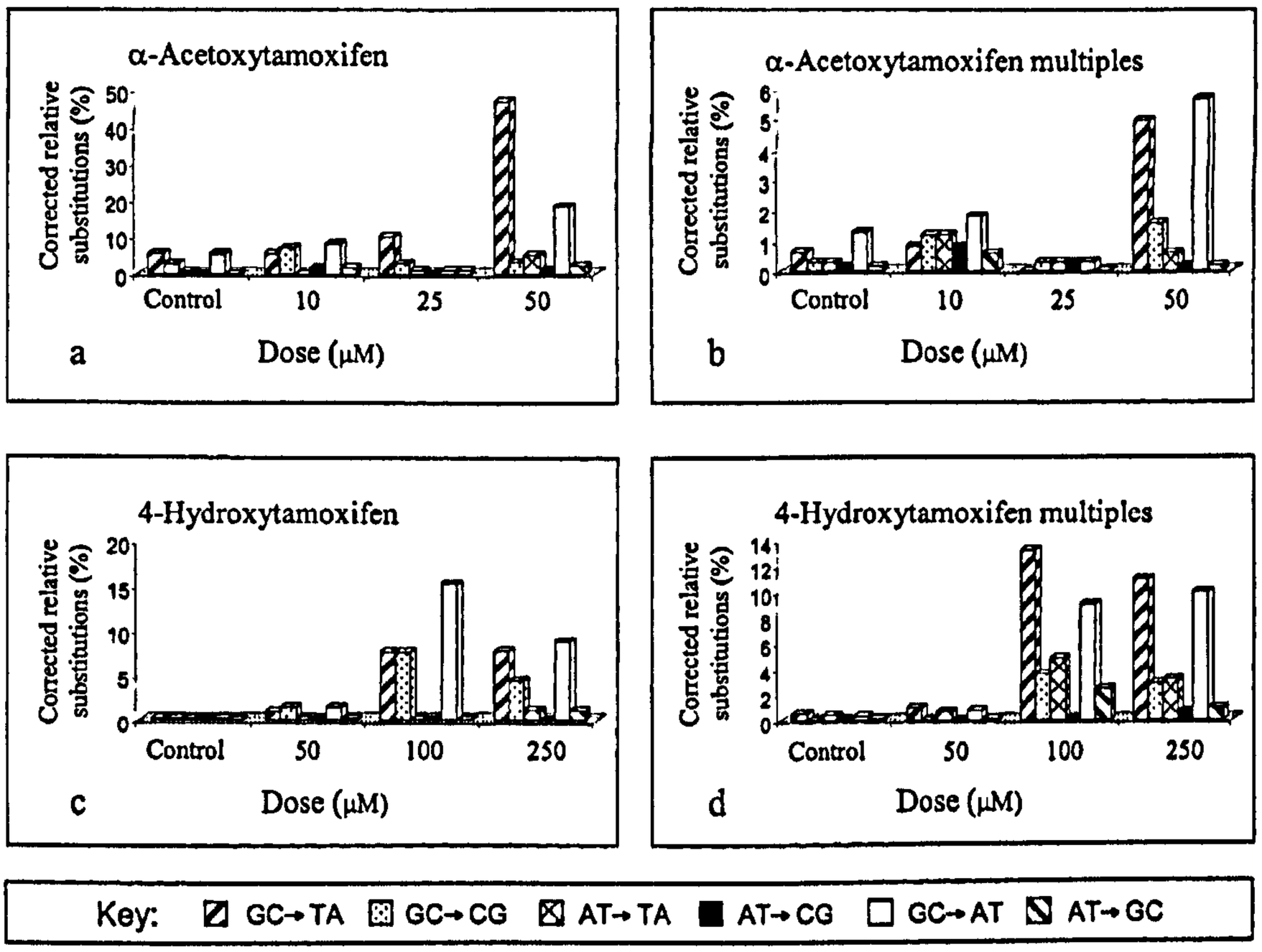

FIGURE 3: Bar charts illustrating the relative amounts of the different base substitutions (corrected for mutation frequency) for pSP189 plasmid treated with α-acetoxytamoxifen (a, single base substitutions; b, multiple substitutions) and 4-hydroxytamoxifen quinone methide (c, single base substitutions; d, multiple substitutions).

DISCUSSION

This paper presents a comparison of the patterns of mutagenesis induced in the *supF* gene by two tamoxifen derivatives, α-acetoxytamoxifen and 4-OHtamQM. α-Acetoxytamoxifen generates the major DNA adducts formed in rat liver (*19*), while the reactive quinone methide of the metabolite 4-hydroxytamoxifen is thought to account for the formation of minor adducts found in rat liver (*23, 24*). Since little is known about the mutagenic potential of tamoxifen adducts other than the major dG-N^2-tam adduct, a primary aim of this study was to evaluate the relative contribution of different types of tamoxifen adducts to the mutagenicity of this drug in human cells. The *supF* gene has been used as the target for mutagenesis by a wide range of mutagens, and, consequently, there is a large database of information regarding the types and distribution of mutations that are induced (*38*–*40*). In this study, we have looked at the mutation spectra induced when treated plasmid was replicated in human adenovirus transformed kidney (Ad293) cells.

Replication of adducted plasmid in human Ad293 cells resulted in an increase in mutation frequency above background control levels for both tamoxifen derivatives. Furthermore, mutation frequency increased with higher plasmid adduct levels, which is in contrast to our earlier study in *E. coli* that reported a lack of correlation between total adduct number on the pLIZ lambda shuttle vector and mutagenicity in the *lacI* gene (*28*). In the present study, for each compound, at the lower doses used there was only a small increase in mutation frequency above the solvent control. With higher doses, inducing above 160 adducts per 10^6 nucleotides (1.6 adducts per plasmid) in α-acetoxytamoxifen-treated plasmid and 8 adducts per 10^6 nucleotides (0.08 adducts per plasmid) in 4-OHtamQM-treated plasmids, there is a sharp increase in mutation frequency. This may suggest there is a threshold level of tolerable damage which the cells are able to repair. Low levels of DNA damage will induce DNA repair, and adducts will be removed. Higher levels may saturate the available repair mechanisms, resulting in an increase in mutation frequency. The fact that 4-OHtamQM induced such a marked increase in mutation frequency in human cells compared to α-acetoxytamoxifen at the two higher treatment doses suggests that the dG-N^2-4-hydroxytamoxifen DNA adduct is a significantly more mutagenic lesion, which may deserve more consideration than it has so far received. The increased mutagenicity of 4-hydroxytamoxifen-induced adducts is thought to be a consequence of the increased hydrogen-bonding potential of dG-N^2-4-OHtam over dG-N^2-tam due to the presence of the 4-hydroxy group (*28*). Extra interactions with complementary bases may disrupt DNA structure, resulting in lesions which could be more readily detected by transcription-coupled error-prone DNA repair or highly inaccurate translesional DNA synthesis, resulting in the incorporation of wrong bases.

Another important feature of the tamoxifen DNA adducts investigated in this study is the low adduct levels needed to induce mutagenesis above background, compared to previous reports for carcinogen adducts. Previously, mutation frequencies similar to those reported here (i.e., around 1×10^{-3}), with 0.05 adducts per plasmid, have been reported for *supF* plasmid containing acetylaminofluorene (*41*), benzo[*a*]pyrene diol epoxide (*42*), or 1,6-dinitropyrene adducts (*43*), replicated in the same cell line (with the same repair proficiency), but containing between 20 and 460 times as many adducts per plasmid as the 4-hydroxytamoxifen. In our experiments,

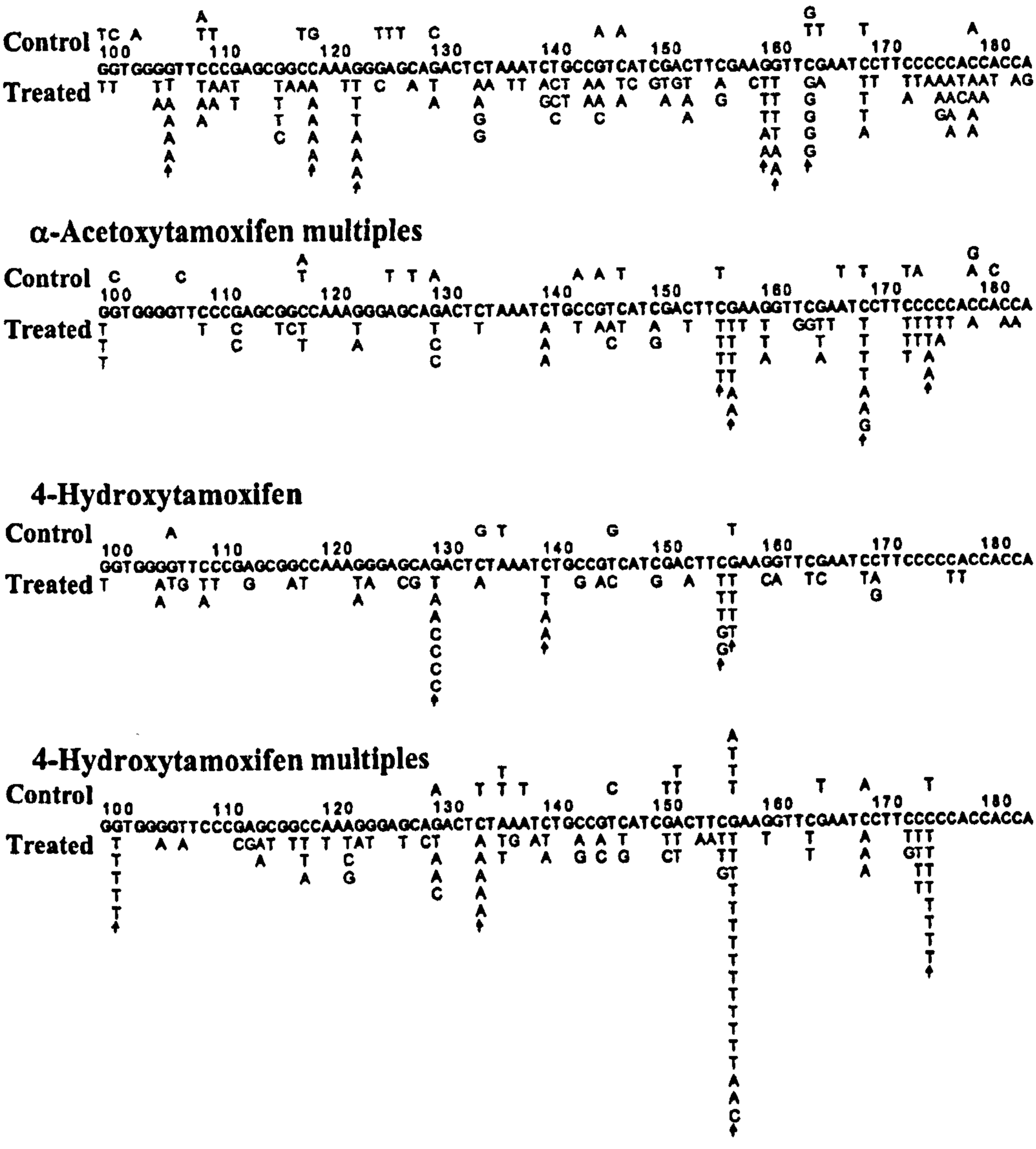

FIGURE 4: Mutation spectra induced in Ad293 cells by α-acetoxytamoxifen and 4-hydroxytamoxifen quinone methide. The 5′ to 3′ sequence of the transcribed strand of the wild-type *supF* gene is shown, with letters below the wild-type sequence indicating the position and type of point mutations induced by treatments and the letters above the wild-type sequence indicating the position and type of point mutations induced in the control samples. The single base substitution spectra comprise all three doses, while multiple base substitutions were from a single dose (50 μM for α-acetoxytamoxifen and 250 μM for 4-hydroxytamoxifen quinone methide). Arrows denote hotspot sites.

α-acetoxytamoxifen showed a similar mutagenicity, on an adduct per plasmid basis, as has been reported for benzo[*a*]pyrene diol epoxide (*42*).

Previously reported mutation spectra in Ad293 cells (*44*, *45*), human lymphoblasts (*46*), and monkey kidney cells (*47*) have shown that the GC → AT transition is the preferred spontaneous mutation. In this investigation, GC → TA transversions are just as prevalent as GC → AT transitions in control plasmids, and GC → CG transversions are only slightly less so. Upon treatment, the GC → AT transition becomes the major mutation for α-acetoxytamoxifen multiple substitutions and 4-OHtamQM single base substitutions. The GC → TA transversion becomes the preferred mutation in α-acetoxytamoxifen single base substitutions and 4-OHtamQM multiple substitutions. Miscoding of the damaged DNA consistent with induction of the GC → TA transversion has been demonstrated for the dG-N^2-tamoxifen adduct in site-specifically-modified oligonucleotides replicated in vitro (*48*). Besides misincorporation of A opposite the dG-N^2-tamoxifen adduct, misincorporation of G and, in some sequences, T was also shown to be possible, albeit at a lower frequency. For both treatments, all hotspots were at GC base pair sites, presumably as a result of adduct formation on deoxyguanosine. There was a noticeable preference for the hotspot site to be preceded and followed by a purine, particularly adenosine. Positions 155, 156, and 174 had hotspots in two or more of the spectra. Hotspots at 156 and 174 appeared in both multiple base substitution spectra, and the hotspot at 156 was present in the 4-OHtamQM single spectra. Position 156 also showed as a hotspot in the control plasmid that was incubated with either acetonitrile or ethanol only. Whether the solvent caused this is not known, although Lewis

et al. did see a hotspot at this position when grouping spontaneous mutations (*49*). Single and multiple base substitutions have been presented on separate spectra because it has been suggested that these multiple mutations arise through a different mechanism to single base mutations (*50*).

The higher percentage of plasmids with multiple mutations induced by 4-OHtamQM compared to α-acetoxytamoxifen may point to a different mechanism of mutagenesis accounting for some of the mutations induced by the former. Similarly, the increased amount of both insertions and large deletions in plasmids treated with 4-OHtamQM compared to α-acetoxytamoxifen suggests that not all mutations induced by 4-OHtamQM are due to misreading of adducted bases, but may be in part due to the formation of cross-links between bases. Previous work has shown that mitomycin C, which reacts with deoxyguanosine at the *N*-2 position like tamoxifen, induces a comparable amount of deletions to 4-OHtamQM in Ad293 cells (34% of all mutations) (*51*) along with a large amount of GC → TA transversions. Mitomycin C is used as a treatment for bladder and rectal cancers due to its DNA cross-linking ability. The anticancer drug Melphalan also causes DNA cross-linking, probably by *N*-7 alkylation of two guanines on opposite DNA strands. When used to treat pZ189 plasmid containing the *supF* gene in Ad293 cells, this also induced a large proportion of deletions (16−28%) (*52*). Metabolic activation of both tamoxifen and 4-hydroxytamoxifen in rat liver microsomes results in the formation of dimers, possibly through a free radical mechanism (*53*). A possible explanation for the induction of large deletions with 4-hydroxytamoxifen-derived adducts, therefore, may be the generation of free radical species resulting in the production of tamoxifen dimer adducts which could be in the form of inter- or intrastrand cross-links. It has previously been reported that multiple mutations correlate with increased levels of single strand breaks in plasmid DNA, induced either by treatment or during repair of induced lesions in repair-competent cells (*54*). The production of free radical species could, therefore, account for this increase in both multiple mutations and deletions, with treatment by 4-OHtamQM, via the induction of strand breaks. To test these hypotheses, we are presently investigating the formation of cross-links and free radical species and their relationship with the induction of strand breaks, and deletion mutations.

Since the *supF* target gene is a double-stranded DNA molecule, it is not possible to determine absolutely which strand contained the mutagenic lesion. However, as tamoxifen does not induce significant amounts of damage at cytosine residues and it is known that the dG-N^2-tamoxifen adduct induces GC → TA mutations, it is probable that the majority of GC → TA mutations are due to misincorporation opposite a damaged G. Considering this, it is possible to review the mutation spectrum from the point of view of any possible strand bias, as the coding strand is more efficiently repaired than the noncoding strand (*55, 56*). After treatment with 4-OHtamQM, the number of mutations presumably derived from damage to the transcribed and nontranscribed strands are almost the same (94 vs 103). After treatment with α-acetoxytamoxifen, there is less damage induced in the transcribed strand than in the nontranscribed strand (72 vs 114). This may suggest that adducts derived from α-acetoxytamoxifen are repaired more efficiently than those formed by 4-OHtamQM in this cell line. This is in contrast to previous work from this group that has shown that tamoxifen adducts are removed from rat liver DNA with no detectable difference in the rates of repair of individual adducts (*6*, Martin, E.A., et al., unpublished results). However, using an in vitro human nucleotide excision repair system, site-specific dG-N^2-tamoxifen adduct isomers in oligodeoxyribonucleotides have been shown to be differentially repaired (*57*). Overall, adducts were removed with a poor to moderate efficiency with the cis-forms being removed most efficiently. It is therefore likely that differences in the chemical structure and overall adduct profile will have a significant influence on the repair and consequently mutation spectra of tamoxifen adducts in human cells. To test this hypothesis, we are currently investigating the role of nucleotide excision repair in the mutagenesis of α-acetoxytamoxifen- and 4-OHtamQM-derived adducts.

There is some evidence that tamoxifen adducts are formed in tissues of women taking this effective anti-cancer drug (*7, 8*). Although these adducts may be formed at low levels, we have shown here that tamoxifen DNA adducts may be highly mutagenic if formed. In particular, the adducts formed by 4-hydroxytamoxifen in the *supF* gene are, when replicated in human Ad293 cells, more mutagenic than those formed by α-acetoxytamoxifen (the major tamoxifen-DNA adducts). The two treatments induce markedly different mutation spectra and mutation types. We would conclude that the 4-hydroxytamoxifen metabolite of tamoxifen has potential to cause serious mutagenic damage to DNA. Whether this is a contributing factor in the induction of endometrial cancer in women by tamoxifen remains to be seen.

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Presence of benzo[*a*]pyrene diol epoxide adducts in target DNA leads to an increase in UV-induced DNA single strand breaks and *supF* gene mutations

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Exposure to DNA damaging agents and mutagens often occurs as combinations of agents, or as complex mixtures of chemicals. We found that plasmid DNA adducted with benzo[*a*]pyrene diol epoxide (BPDE) was more susceptible to UV-induced single strand breaks than was control DNA. To determine whether the increase in DNA damage also applied to mutagenic lesions, the *supF* gene forward mutation assay was used to compare mutations induced by BPDE alone, UVB, UVC, BPDE followed by UVB and BPDE followed by UVC. It was found that the mutation frequency for BPDE + UVB (1167 in 10^4 transformants) was higher than BPDE alone (12 in 10^4 transformants) or UVB alone (446 in 10^4 transformants), and the mutation frequency for BPDE + UVC (197 in 10^4 transformants) was higher than BPDE alone or UVC alone (26 in 10^4 transformants). For BPDE + UVB and BPDE + UVC there was a significant increase in plasmids with multiple mutations. Whilst these indicate error prone repair due to the single strand breaks, the different mutation frequencies in plasmids treated to give similar levels of strand breaks suggest other mechanisms for the mutations in plasmids with single mutation events. The spectrum of non-multiple mutations in the two combined treatments included both UV signature mutations (GC→AT as the most common mutation) and BPDE signature mutations (GC→TA and GC→CG as the most common mutations). However, the increase in absolute mutation frequency of BPDE signature mutations between BPDE treatment and BPDE + UV treatment was greater than the increase in absolute mutation frequency of UV signature mutations, even though the level of BPDE adducts was identical in each case. These results suggest two possibilities: (i) the BPDE adducts are photoactivated to a more mutagenic lesion, or (ii) the presence of UV lesions lead to the BPDE adducts becoming more mutagenic.

Introduction

Mutagenic DNA lesions can arise as the result of the interaction of DNA with a myriad of agents that are derived, *in vivo*, exogenously and/or endogenously. In many cases, exposure to mutagenic agents occurs in the form of combined exposures or complex mixtures of mutagens e.g. cigarette smoke or diesel exhaust emissions (1). In experimental studies it is often desirable to reduce exposure to the level of the individual agent, in order to elucidate the processes involved. However, it is possible that the induction of damage and mutations by a specific agent may be altered if the exposure occurs in combination with another agent or as a mixture of agents. For example, it has been demonstrated that some carcinogenic compounds that normally require metabolic activation to reactive species before binding to DNA occurs can be photoactivated to direct acting mutagens by UVA irradiation (2–5). As well as interactions between agents prior to DNA binding, which may act to modulate either the binding of genotoxic agents or the mutagenicity of the reactive species, it may be possible that the mutagenicity of bound adducts is modified by other DNA reactive agents, or by the presence of other lesions on the DNA molecule. As a first step in addressing this possibility we have combined UV irradiation with adduction by benzo[*a*]pyrene diol epoxide (BPDE) as the DNA damaging events. BPDE is the ultimate carcinogen formed by the metabolism of the polycyclic aromatic hydrocarbon (PAH), benzo[*a*]pyrene, which is found in cigarette smoke, burnt food and smoke from the burning of fossil fuels. BPDE binds mainly to guanines (6) and is known to induce primarily GC→TA transversions (7). UV irradiation, a known mutagen and carcinogen, induces GC→AT transitions, mainly through the formation of cyclopyrimidine dimers and (6–4)photoproducts (8–11). Evidence that PAH and UV exposure (in sunlight) may have a combined toxic effect *in vivo* has come from studies on the phototoxicity of PAH in aquatic organisms (12–14). In terms of human carcinogenesis, a combination effect of UV irradiation and PAH could be postulated in situations where occupational exposure to PAH is combined with regular exposure to high levels of sunlight, such as for road maintenance workers (15) or in medicinal therapies for psoriasis that combine coal tar application and UV irradiation (16).

Amongst the different types of DNA lesions that are induced by UV irradiation, DNA single strand breaks may be easily monitored using a plasmid strand break assay, as the introduction of a single strand break into supercoiled plasmid DNA results in the conversion of the supercoiled plasmid into relaxed or nicked plasmid, which migrates more slowly than supercoiled plasmid upon agarose gel electrophoresis (17). A versatile method for analysing induced mutations is the *supF* forward mutation assay (18), which has been applied to the study of a wide range of mutagens (10,18–21). Using the *supF* mutation assay, information can be obtained as to the type of mutations induced by a compound and the distribution of these mutations within the target gene.

In this pilot study, we have investigated the effects of UV irradiation on DNA that has been adducted *in vitro* with BPDE, by measuring DNA single strand breaks in a plasmid strand

Abbreviations: BPDE, benzo[*a*]pyrene diol epoxide; PAH, polycyclic aromatic hydrocarbon; HRP, horseradish peroxidase.

break assay and the effect of the presence of BPDE adducts in the target DNA on UV induced mutations in the *supF* gene forward mutation assay (10).

Materials and methods

Induction of DNA damage

Plasmid DNA (pUC18, 20 μg in 20 μl water) was incubated with either acetone (20 μl) or BPDE dissolved in 20 μl acetone (approximately 10 μM) for 10 min at 37°C. Unreacted BPDE was removed by extraction into ethyl acetate. DNA was precipitated by addition of 0.1 vol 2.5 M sodium acetate, pH 5 and 2.5 vol ethanol, followed by storage at −20°C, overnight. Precipitated DNA was pelleted and washed twice with 70% (v/v) ethanol. BPDE adduction levels were determined by ^{32}P-post-labelling assay, as described previously (22) except that visualization and quantification of adducts was performed using a PhosphorImager (Molecular Dynamics, Sevenoaks, Kent) with ImageQuant software, version 3.3. For UV irradiation, DNA was re-dissolved in water (0.2 μg/μl) and 50 μl DNA solution was pipetted onto the surface of a plastic Petri dish. The DNA was irradiated with a UV lamp at a dose rate of 0.2 mW/cm^2 for UVB and 0.7 mW/cm^2 for UVC. At set time points (0, 10, 20, 30, 40, 50 and 60 min for UVB and 0, 0.75, 1.5, 2, 3, 4 and 5 min for UVC), 5 μl aliquots were removed and added to 20 μl gel loading buffer (0.05% bromophenol blue, 0.05% xylene cyanol, 6% glycerol in water) and stored on ice. Upon completion of the dosing regime, samples were analysed by agarose gel electrophoresis for single strand breaks (see below).

For the *supF* mutagenesis experiments the same procedures as above were performed using the pSP189 plasmid, except that one UV exposure was used for each wavelength. For UVB irradiation, the plasmid samples were irradiated for 20 min (giving a dose of 2.4 kJ/m^2), whilst for UVC irradiation, the plasmid samples were irradiated for 1.5 min (giving a dose of 0.63 kJ/m^2). After irradiation plasmid samples were stored at 4°C until transfection into Ad293 cells, which occurred within 24 h of irradiation.

Single strand break assay

After UV irradiation DNA samples were loaded onto a 0.8% (w/v) agarose gel made up with 1× TBE (90 mM Tris–borate, 1 mM EDTA, pH 8.0), on which supercoiled and relaxed plasmid were separated by electrophoresis at 12 V, overnight, with 1× TBE as running buffer. Following electrophoresis the gel was stained in ethidium bromide (0.5 μg/ml) for 1 h followed by destaining in water for 1 h. DNA bands were visualized and quantified using a Biorad MultiImager (BioRad, Hercules, CA). The amount of DNA in the supercoiled and relaxed forms of the plasmid was estimated by integration of the intensity of the pixels comprising each band. As supercoiled DNA binds lower amounts of ethidium bromide compared with open circular DNA, the intensities of supercoiled bands were adjusted by a factor of 1.4 (17).

SupF mutation assay

Subconfluent Ad293 cells (an adenovirus transformed embryonic kidney cell line, which was a kind gift from Tony Dipple, NCI–FCRDC, Frederick, MD) were transfected with control or treated plasmid (10 μg per 9 cm culture plate) using the calcium phosphate precipitation technique (23). After 48 h, plasmid was recovered using a Qiagen plasmid purification kit (Qiagen, Crawley, West Sussex). Aliquots of recovered plasmid were used to transform electrocompetent MBM7070 *Escherichia coli* by electroporation using a Gene Pulser apparatus (BioRad). Transformants were plated onto LB agar plates containing 100 μg/ml ampicillin, 75 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactose (Xgal) and 25 μg/ml isopropyl-β-D-thiogalactoside (IPTG).

Plasmid was extracted from white and pale blue mutant colonies and sequenced using the primer 5′-GGCGACACGGAAATGTTGAA-3′ (Protein and Nucleic Acid Chemistry Laboratory, CMHT, University of Leicester). The shuttle vector pSP189 contains an eight base 'signature sequence' giving 4^8 (65 536) possible unique sequences (note that this figure differs from that of 2×4^8 given in the original reference (10), as we do not believe that insertion of the sequence in either direction doubles the number of possible sequences). Any mutants with a duplicated 'signature' were excluded from further analysis. Poisson distribution analysis was used to assess the randomness of spectra. Hotspots were assumed when >5% of total mutations occurred at a single point in the spectrum.

Results

Induction of DNA single strand breaks in plasmid DNA

Supercoiled plasmid (pUC18) that had been treated with BPDE in acetone, or acetone alone (control), was irradiated with up to 7.2 kJ/m^2 UVB or up to 2.1 kJ/m^2 UVC. The BPDE adduct level in the treated DNA was determined by ^{32}P-post-labelling to be about 1 adduct in 10^3 nucleotides (data not shown). The UVB and UVC doses used were chosen from preliminary range-finding experiments with untreated plasmid (data not shown), as doses that gave measurable increases in the amount of DNA in the form II plasmid band (relaxed circular plasmid containing at least one strand break), without inducing significant levels of linear DNA. After determination of the relative amount of DNA in the form I and form II bands, the data was plotted as the negative natural logarithm of the fraction of form I DNA remaining at each dose, against time of exposure (Figure 1). Plotting the results in this way is equivalent to plotting the average number of single strand breaks per plasmid molecule versus UV dose. The UV dose at which there were on average, one single strand break per plasmid molecule (D_0) can be read from these plots, or calculated from the reciprocal of the slope. From Figure 1 it can be seen that D_0 for BPDE + UVB and UVB control were 5.9 and 24.5 kJ/m^2, respectively, whilst D_0 for BPDE + UVC and UVC control were 1.9 and 10.9 kJ/m^2, respectively. In other words, there was a greater number of UV-induced single strand breaks introduced into the plasmid DNA that had been adducted with BPDE, compared with the control plasmid, at any given dose of either UVB or UVC irradiation.

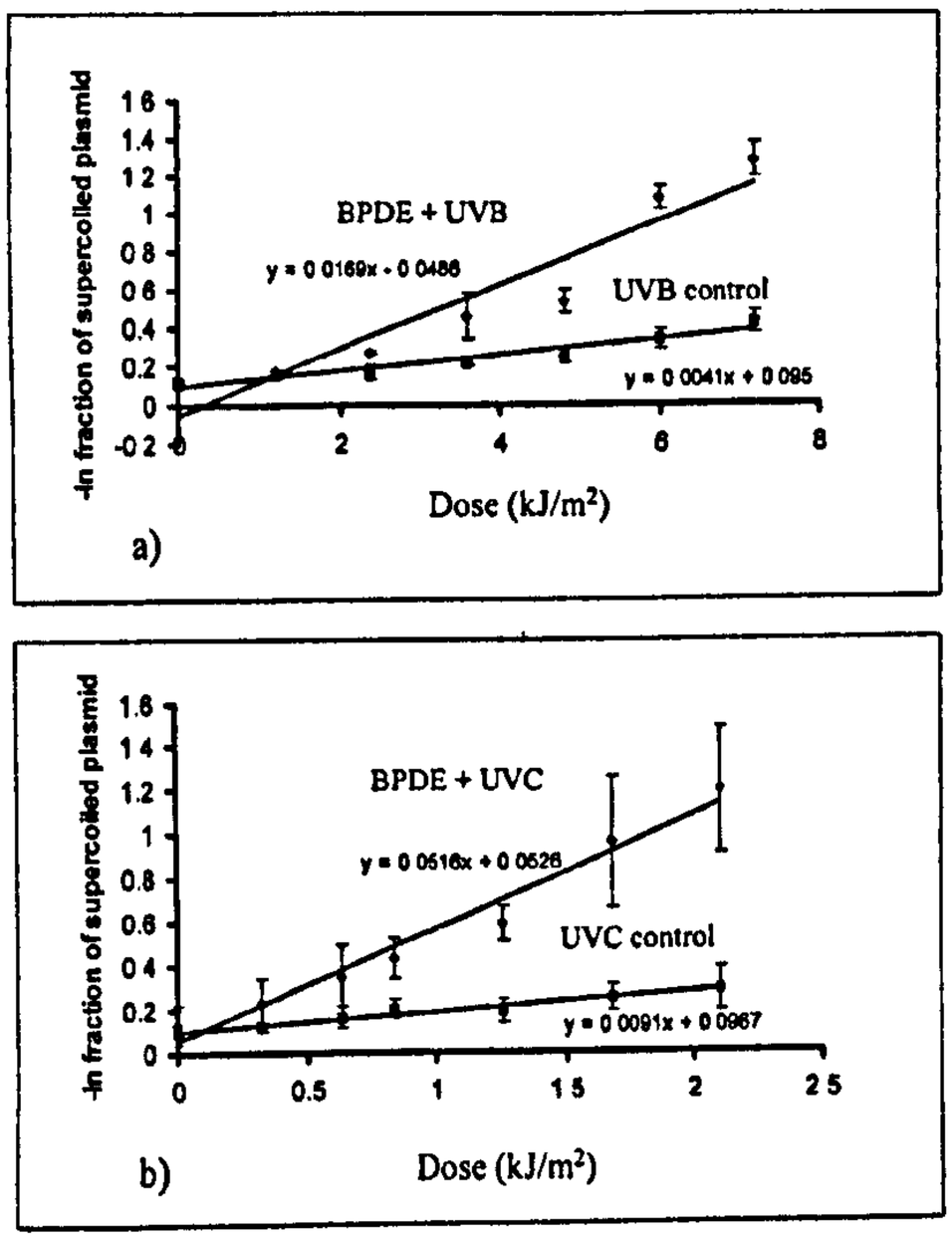

Fig. 1. Induction of single strand breaks in plasmid DNA. Control pUC18 DNA and BPDE adducted pUC18 DNA was irradiated with (a) UVB, or (b) UVC. Supercoiled (form I) plasmid was separated from open circular (form II) plasmid by agarose gel electrophoresis. The reduction in the fraction of supercoiled DNA with increasing time of exposure was calculated and the negative natural logarithm of this fraction plotted against time of UV irradiation. The values on the *y* axis are equivalent to the average number of single strand breaks per plasmid molecule.

Mutation frequency in supF assay

Aliquots of six pSP189 samples, untreated control (control), BPDE treated (BPDE), plasmid irradiated with UVB (UVB), BPDE treated plasmid that was subsequently irradiated with UVB (BPDE + UVB), plasmid irradiated with UVC (UVC)

Table I. Mutation frequency induced by the various treatments of pSP189

Treatment	Colonies screened	Mutation frequency[a]	Plasmids with multiple mutations[b] (%)
Control	216 000	0.3	0
BPDE[c]	94 000	12	0
UVB	3000	446	5
BPDE + UVB	3000	1167	22
UVC	18 000	26	0
BPDE + UVC	13 000	197	17

[a]Frequency of mutants per 10^4 transformants.
[b]Multiple mutations are two or more non-adjacent mutations that occur in the same plasmid.
[c]BPDE-treated plasmid contained 2.2 adducts per 10^4 nucleotides, as determined by ^{32}P-post-labelling analysis (about one adduct per plasmid).

and BPDE treated plasmid that was subsequently irradiated with UVC (BPDE + UVC), were transfected into Ad293 cells to allow for mutagenesis to occur within the human cell line. The UVC dose of 0.63 kJ/m^2 was chosen based on results obtained in preliminary *supF* assay experiments in this laboratory (unpublished results), whilst the UVB dose (2.4 kJ/m^2) was selected on the basis of this dose of UVB irradiation inducing a similar level of conversion of supercoiled to relaxed plasmid as was induced by the 0.63 kJ/m^2 dose of UVC irradiation, as determined in the plasmid single strand break assay (Figure 1). Extracted plasmid that had been replicated in the Ad293 cells was screened for the presence of mutant *supF* gene by transformation into *E.coli* MBM7070. Table I shows the mutation frequency that was observed in these experiments. It can be seen from the control plasmid that the background mutation frequency was suitably low, at 3 mutants in 10^5 transformants. For all other treatments mutation frequency was significantly enhanced. The level of BPDE adducts in the BPDE treated pSP189 was determined by ^{32}P-post-labelling to be about 2.2 adducts in 10^4 nucleotides, which is the equivalent of about 1 adduct per plasmid molecule. In this experiment, this has led to a mutation frequency of 12 mutants per 10^4 transformants (Table I). It can be noted as well, that the presence of BPDE damage in the plasmid led to an increase in mutation frequency upon UV irradiation with UVB or UVC that was greater than predicted from the mutation frequency of either treatment alone. For example, the mutation frequency for BPDE + UVB was 1167 in 10^4 transformants, which was ~2.5-fold greater than the addition of the BPDE mutation frequency (12 in 10^4 transformants) and the UVB mutation frequency (446 in 10^4 transformants). Similarly, the mutation frequency for BPDE + UVC (197 in 10^4 transformants) was ~5-fold greater than the additive mutation frequencies for BPDE (12 in 10^4 transformants) and UVC alone (26 in 10^4 transformants). When mutant plasmids were sequenced it was found that two classes of mutated plasmid were present: those containing a single or tandem mutation in the *supF* gene and those containing two or more non-adjacent (multiple) mutations. The frequency of multiple mutations was greatest in the combined treatments (Table I), with 20 out of 91 sequenced BPDE + UVB plasmids containing a total of 46 multiple mutations and 11 out of 64 sequenced BPDE + UVC plasmids containing a total of 27 multiple mutations. The only other protocol to yield multiple mutations was UVB treated plasmid, for which 3 out of 66 sequenced plasmids contained six multiple mutations. Interestingly, two of these plasmids contained identical multiple mutations, AT→GC at site 120 and GC→AT at site 124.

Table II. Percentage single base substitution mutations[a] induced by the various treatments of pSP189[b]

	BPDE	UVB[b]	BPDE + UVB (multiples)[c]	UVC	BPDE + UVC (multiples)
GC→AT	11	84	77 (56)	79	59 (48)
GC→TA	65	7	14 (16)	8	24 (18)
GC→CG	22	0	2 (12)	8	10 (6)
AT→GC	2	0	3 (8)	3	5 (12)
AT→CG	0	6	0 (6)	0	0 (0)
AT→TA	0	3	5 (2)	3	2 (15)

[a]Percentages have been rounded off, which means that numbers in the columns do not always add up to 100. Single base substitutions include tandem mutations. In addition to base substitutions, there were two deletions in the sequenced UVB treated plasmids and three deletions in the sequenced BPDE + UVC treated plasmids.
[b]There were also six multiple mutations sequenced for the UVB treated plasmid, too few to represent as percentages. These were three AT→GC, two GC→AT and one GC→TA.
[c]Percentage of multiple mutation types, as a separate class, are shown in parentheses.

Table III. Absolute mutation frequency (per 10^4 transformants) for mutations at GC pairs

Mutation	BPDE	UVB	BPDE + UVB	UVC	BPDE + UVC
GC→AT	1.3	375	899	21	116
GC→TA	7.8	31	163	2.1	47
GC→CG	2.6	0	23	2.1	20

Mutation spectra in supF assay

Mutant colonies were collected from the different transformations and sequenced to determine the types and distribution of induced mutations in the *supF* gene. It can be seen from Table II that in all treatments induced mutations were mainly at GC pairs. There were two classes of mutant plasmid, those that contained single or tandem mutations and those that contained multiple mutations. The types of mutations for these two classes of mutant plasmid (singles and multiples) are shown separately in Table II, as it has been shown that multiple mutations arise by a separate mechanism to single or tandem mutations (24), a mechanism that involves the induction of error prone replication (25). For the singles class of mutants, the most common base substitution mutation induced by BPDE was the GC→TA transversion (65%), followed by GC→CG transversions. For all the treatments involving UV irradiation, the GC→AT mutation was the most common (84% for UVB and 79% for UVC). However, it can be seen that the percentage of both GC→TA and GC→CG mutations was higher in BPDE + UVB compared with UVB, and BPDE + UVC compared with UVC. It can also be seen from Table II that the percentage of GC→AT mutations is lower in the multiple class mutants compared with the single class mutants for both BPDE + UVB and BPDE + UVC. For BPDE + UVB this is due to an increase in the percentage of GC→TA, GC→CG, AT→GC and AT→CG mutations, whereas for BPDE + UVC it is due to an increase in the percentage of AT→GC and AT→TA mutations.

Table III shows the types of single class base substitution mutations that were induced at GC base pairs, expressed in

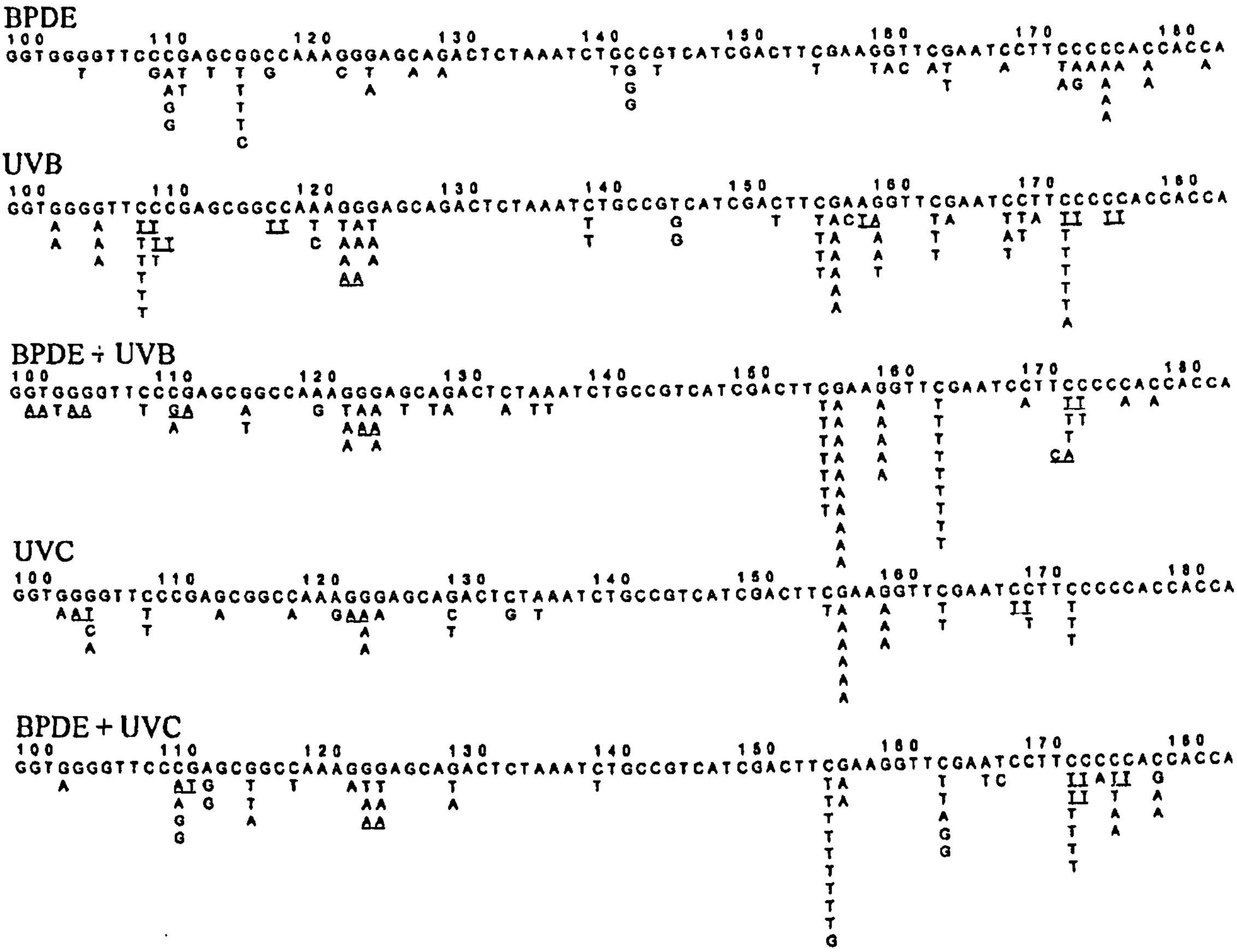

Fig. 2. Mutation spectra induced by the various treatments used. The 5′ to 3′ sequence of the transcribed strand of the wild-type *supF* gene is shown, with letters below the wild-type sequence indicating the position and type of point mutations induced by the various treatments.

terms of the absolute frequency of these mutations. By expressing the results in this way it can be seen more clearly that the increase in mutation frequency of different types of GC base substitutions varies. Hence, the increase in frequency of GC→AT mutations is from 375 to 899 in 10^4 transformants in UVB compared with BPDE + UVB samples, whereas the increase in frequency of GC→TA mutations in the same samples is much greater, going from 31 to 163 in 10^4 transformants. This is an increase of 5.3-fold for GC→TA mutations, compared with an increase of 2.4-fold for GC→AT mutations in these samples. Similarly, for the UVC treated samples, the increase in GC→TA transversions (22-fold) and GC→CG transversions (10-fold) in BPDE + UVC versus UVC samples was greater than the increase in GC→AT transitions between the two samples (6-fold).

The distribution of mutations (excluding multiple mutations) within the *supF* gene for the five treatments is shown in Figure 2. The distribution spectra of the multiple mutations have been shown separately (Figure 3). In each spectrum of mutations there are hotspots for mutation. Examination of the spectra in Figure 2 reveals some differences in the distribution of these hotspots, particularly between the BPDE spectrum and the UV-induced spectra. For the BPDE treated spectrum there are clusters of mutations between sites 109–115 and sites 172–178. In particular hotspots of mutation are seen at sites 110, 115, 142 and 175. The UVB spectrum contains hotspots at 108, 122, 155, 156, 159 and 172, whilst the UVC spectrum contains hotspots at 104, 123, 156, 159 and 172. When comparing the BPDE + UVC spectrum to the BPDE and UVC spectra it can be seen that the BPDE + UVC spectrum contains aspects of both individual spectra. The BPDE-induced hotspot at site 110 is present, as is the UVC hotspot at 172. Interestingly, the strong GC→AT mutation hotspot at site 156 (a G in the transcribed strand of *supF*) in the UVC spectrum has switched to a strong GC→AT mutation hotspot at site 155 (a C in the transcribed strand) in the BPDE + UVC spectrum. Site 163, which was not a hotspot in either BPDE or UVC spectra, appears as a hotspot, with both UV consistent GC→AT mutations and BPDE consistent GC→TA and GC→CG mutations at this site in the BPDE + UVC spectrum.

Figure 3 shows the spectra of multiple mutations observed for the BPDE + UVB and BPDE + UVC protocols. For ease of comparison, the single mutation spectra for these protocols are reproduced in this figure. For the BPDE + UVB multiple spectrum it can be seen that there were hotspots of mutation at sites 155, 156 and 163, which were also hotspots in the BPDE + UVB single mutation spectrum, and at site 124, which was not classified as a hotspot in the single mutation

Fig. 3. A comparison of the spectra of single and tandem mutations with the spectra of multiple mutations (two or more non-adjacent mutations in a sequenced plasmid) for the BPDE + UVB and BPDE + UVC treatments.

spectrum. A notable hotspot at site 159 in the BPDE + UVB single mutation spectrum was not present in the multiple spectrum. Comparing the BPDE + UVC single and multiple mutation spectra it can be seen that the only common hotspots occur at sites 115 and 172.

Discussion

The majority of *in vivo* exposure to DNA damaging agents occurs as a result of interactions of complex mixtures of chemicals, such as those found in cigarette smoke or diesel exhaust emissions, with DNA (1). As well as complex mixtures, combined exposures to agents from different sources may also occur, for example bitumen and sunlight (15). When studying DNA damage and mutagenesis, it is often necessary to reduce this complex situation to a more simple single-agent exposure system, and the types of DNA damage and mutations induced by specific agents have been studied in a wide number of experimental systems. To start to address the question of what the effect of combined exposures may be on individual DNA damaging agents, we have investigated the combination of two mutagens that have been extensively studied: BPDE, a chemical carcinogen that forms well characterized bulky DNA adducts, and UV irradiation, a physical mutagen that induces cyclopyrimidine dimers and (6–4)photoproducts. The reason for choosing this combination was twofold. Firstly, as an experimental system the types of mutations induced by BPDE and UV irradiation are quite distinct, with the most common mutation induced by BPDE in a number of mutation assays being GC→TA transversions (7,26), whilst GC→AT mutations are the most common base substitutions induced by UV irradiation (9,27). These mutations arise as a result of damage to purines (chiefly guanine) in the case of BPDE (6,7) and pyrimidines in the case of UV (11,28). Hence, it would be possible to distinguish between the mutagenic contributions of the two agents in a sequential exposure protocol. (In this paper, GC→TA mutations are referred to as BPDE signature mutations, whilst GC→AT mutations are referred to as UV signature mutations as a short hand term to distinguish between the likely source of these mutations in these experiments). Secondly, there is evidence that in aquatic organisms, the toxicity of PAH pollutants is enhanced by UV irradiation (12–14). There is, therefore, some rationale to thinking that DNA damaged by a PAH such as BPDE may be more susceptible to UV induced damage or mutations.

One type of damage induced in DNA by UV irradiation is the single strand break, and the induction of single strand breaks can be easily analysed using a plasmid strand break assay. In our system, the level of UV-induced single strand breaks was higher for any given UV dose when the plasmid was adducted with BPDE at a level of ~1 adduct in 10^3 nucleotides, than when it was not adducted. This increased susceptibility of BPDE adducted DNA to UV-induced strand breaks applied to both UVB and UVC irradiation. Whilst in cells there is evidence that some UVB-induced strand breaks may be mediated by repair enzymes (29), in the cell-free system studied here all strand breaks must be induced directly, or by production of reactive oxygen species in the irradiated DNA solution. It is possible that the BPDE adduct acts as a site of preferential induction of strand breaks, or that the presence of the BPDE adduct is involved in the UV-induced generation of reactive oxygen species from water molecules, and it is these reactive oxygen species that induce the strand breaks. It has previously been shown that laser pulse irradiation at 355 nm of site-specifically modified BPDE-guanine oligo-

nucleotides induces strand cleavage at, and to a lesser extent near to, the adducted base, by a mechanism that involves either photo-induced electron transfer or the production of a local free radical that ultimately results in strand breaks (30,31).

DNA single strand breaks are formed by UVB and UVC at much lower levels than are mutagenic cyclopyrimidine dimers and 6–4 photoproducts (32). In order to investigate whether mutagenicity was increased when BPDE adducts were present on plasmid DNA prior to UV irradiation, we repeated the exposures using the pSP189 plasmid that carries the *supF* target gene as the substrate DNA and studied the induced mutations of the different treatments. Mutations induced by BPDE and UV irradiation in separate exposures have previously been reported in this system (7,10). Based on the quantification of BPDE adducts by the ^{32}P-post-labelling assay, our BPDE-induced mutation frequency was ~5-fold higher at a similar adduct level (about one adduct per plasmid molecule, on average) than that reported by Maher *et al.* (7). The spectrum of mutation types was similar in the two studies with GC→TA, GC→AT and GC→CG mutations accounting for 63, 9 and 18%, respectively, in the earlier study (7) and for 65, 11 and 22%, respectively, in this study. The distribution of mutations, however, was quite different, with no common hotspots of mutation observed in the two spectra. The sites of hotspot for mutation in the current study, 110, 115, 142 and 175, were not hotspots in the previous study. Interestingly, the hotspots in the current study all correlate with hotspots for BPDE adduction in the *supF* gene, as determined by a polymerase stop assay in a previous study (7). Maher *et al.* (7) showed that hotspots of damage and hotspots of mutation did not correlate. However, as we did not perform the polymerase stop assay here, we cannot comment on whether our results disagree with the previous finding, but merely point this finding out as an interesting observation. Differences in the distribution of mutations between this study and Maher *et al.* (7) are probably due to the fact that some of the plasmid sequence in pSP189 differs from that of pZ189 used in the earlier study. Such differences in induced mutation spectra in pS189 (an earlier version of pSP189) and pZ189 have been reported for aflatoxin B_1 (33) and it is known that alterations in DNA sequence some distance from the target gene can modulate mutational spectra (34,35).

In our experiments, the most notable effect on UV-induced mutagenicity when the DNA was already adducted with BPDE was to increase the mutation frequency of the combination treatment compared with UV alone, for both UVB and UVC. For UVB and UVC the mutation frequency was 2.6- and 7.6-fold higher, respectively, when the plasmid was already adducted with BPDE than when it was not. The effect of the combined treatment on mutation frequency was more than just additive, as the mutation frequency of the BPDE adducted plasmid was lower than the mutation frequency of either UV irradiation treatment. Hence, the observed increase in one type of measure of DNA damage, the single strand break, was reflected by an increase in mutation frequency when the target DNA was already modified with BPDE.

Analysis of the mutation spectra for the combined treatments shows an increase in the percentage of GC→TA and GC→CG mutations in the BPDE + UV treatments compared with UV alone, which is expected. However, when the absolute mutation frequency for each type of base substitution is considered (Table III), the increase in GC→TA and GC→CG mutations in the BPDE + UV samples compared with UV alone is higher than the increase in GC→AT mutations. For example, the mutation frequency for GC→AT mutations increases just over 2-fold for UVB and almost 6-fold for UVC when the BPDE adducts are present, whereas the increase in GC→TA mutations is over 5- and 22-fold, respectively, for UVB and UVC. It appears that the mutations induced by UV treatment of BPDE-adducted plasmid include a greater frequency of BPDE signature mutations (GC→TA, GC→CG) as well as a greater frequency of UV signature mutations (GC→AT), and that the level of increase in the BPDE signature mutations is higher than the level of increase in the UV signature mutations. This is important because the level of BPDE adducts is the same in both cases, but the subsequent exposure of BPDE plasmid to UV has led to a greater increase in a type of mutations associated with BPDE adducts, compared with the increase in the mutations associated with UV adducts. For the UVC treatment, the relative increase of apparent BPDE signature mutations is also reflected by the presence of a hotspot of GC→TA and GC→CG transversions at site 110 in the BPDE + UVC spectrum. There is a hotspot at this site in the BPDE spectrum, but not in the UVC spectrum. This implies that the hotspot is related to BPDE damage. Previous studies of PAH mutagenicity in the *supF* gene have shown that whilst the hotspots of damage and mutation do not correlate, mutations are always targeted to bases that are adducted (20).

It has previously been shown that compounds that normally require enzyme mediated activation to be mutagenic in bacterial mutagenicity assays, such as aflatoxin, dimethylbenzo[*a*]anthracene and *N*-nitrosodimethylamine, are direct acting mutagens when exposed to UVA radiation or sunlight (2–5). For *N*-nitrosodimethylamine, the formation of O^6-methylguanine and N7-methylguanine, DNA lesions known to be formed by activated metabolites of *N*-nitrosodimethylamine, as well as 8-oxo-deoxyguanosine, an oxidative lesion formed as a result of UVA irradiation, were detected (5). In a study using *N*-nitrosopyrrolidine it was shown that the compound was a direct acting mutagen in M13mp2 phage in the presence of UVA radiation, with DNA lesions formed by *N*-nitroso-1-phosphonooxypyrrolidine, a photoactivation product of *N*-nitrosopyrrolidine (36). In these studies it should be noted that UVA irradiation, and in the case of activation by sunlight possibly UVB irradiation also, increases binding of the mutagen to DNA by activation of the parent compound to a reactive intermediate that binds to DNA, whereas in the experiments we describe, the BPDE adducts are already bound to the DNA at the time of UV irradiation. In terms of the induction of DNA strand breaks, it has previously been shown in experiments using site-specifically BPDE modified oligonucleotides that the presence of non-bound benzo[*a*]pyrene tetraols also led to photoinduced strand cleavage of the oligonucleotide, albeit at a 7-fold higher tetraol concentration for a response equivalent to the BPDE–DNA oligonucleotide (30).

The explanation for the increased mutagenicity in UV irradiated BPDE-adducted plasmid may be that this increased mutagenicity is the direct result of the increase in single strand breaks shown by the plasmid strand break experiments. Although the single strand breaks are not miscoding lesions, it has previously been reported that the introduction of single strand breaks into the plasmid pZ189 (to yield nicked plasmid) led to an increase in mutation frequency over background in a repair deficient cell line (25). Furthermore, the presence of multiple mutations within a single plasmid was found to be

associated with the presence of nicked plasmid. Such multiple mutations also occurred when UV irradiated plasmid was transformed into repair proficient cells. Thus, the presence of nicks in transformed plasmid, or the repair of UV damaged plasmid (during which nicks may be formed), was associated with an error prone repair replication that accounted for some of the observed mutations (25). Although the use of different plasmid and cell types between the experiments reported here and the earlier experiments (in which spontaneous mutation frequency was higher) makes direct comparison of the results difficult, it seems likely that the increase in levels of UV-induced single strand breaks, as shown in the plasmid strand break assay experiments, has contributed to the increased mutation frequency. The presence of increased strand breaks certainly correlates with the higher levels of multiple mutations in the BPDE + UVB or BPDE + UVC treated samples compared with the other samples. Nevertheless, it is unlikely that this is the only explanation for the increase in mutation frequency. In our mutation experiments the doses of UVB and UVC used were selected because they gave similar levels of strand breaks in the plasmid strand break assay, not because they gave similar levels of mutation. Although this means that the UVB and UVC mutation frequency results cannot be directly compared, it also means that plasmid with different UV treatments but similar levels of single strand breaks gave very different mutation frequencies. This would not be predicted if the induction of single strand breaks was entirely responsible for the increase in mutation frequency.

We consider there to be four other possible explanations for the increased mutation frequency when BPDE adducts are present: (i) GC→TA mutations are induced by oxidative DNA damage, and the production of reactive oxygen species as a result of an interaction between the BPDE adduct and UV radiation could account for increased mutation frequency (a mechanism similar to that by which non-bound benzo[*a*]pyrene tetraol enhanced photoinduced cleavage of BPDE-modified oligonucleotides as discussed above). (ii) The presence of the BPDE adducts may cause the DNA to absorb more energy from the UV radiation, leading to enhanced UV damage, which could be targeted around the sites of BPDE adduction. (iii) UV irradiation of the BPDE adduct may produce a photoactivation product that is inherently more mutagenic than the original adduct. (iv) The presence of UV and BPDE adducts in the same DNA molecule alters the mutagenicity of one or the other.

Looking at the mutation spectra generated in this study, it would appear that in the BPDE + UV spectra, the BPDE adducts have contributed to the increased frequency of mutations in the combination treatment of BPDE + UV versus BPDE alone, even though the level of BPDE adducts has not altered. This evidence suggests that the BPDE adducts have themselves become more mutagenic, either due to photoactivation of the adduct, or to effects of UV adducts nearby (i.e. a change in DNA conformation caused by the presence of a UV adduct has led to a BPDE adduct becoming more mutagenic than before). The question of what factors may influence the formation of different mutagenic spectra by the same adduct forming agent, or even by the same specific adduct, under different circumstances has been addressed in elegant detail by Rodriguez and Loechler (34,37) and Seo *et al.* (38), with particular reference to adducts formed by BPDE. They have provided a strong case that the same adduct [specifically the major adduct of (+)-anti-BPDE formed by trans addition of N^2-dG to C10 of (+)-anti-BPDE] can be locked into two conformations, one that leads to G→T mutation, and another that leads to G→A mutation. Perhaps the outcome of the UV irradiation of BPDE adducted DNA is to induce an analogous conformational change in the adducts?

Whilst our results may relate specifically to the interaction of UV irradiation with BPDE adducted DNA, this work was inspired by a general interest in the possible mutagenic effects of combined exposures that may result from the presence of more than one type of mutagenic lesion in DNA. By examination of data from an earlier study of the mutagenicity of the tamoxifen derivatives, α-acetoxytamoxifen and 4-hydroxytamoxifen (39), we note another potential example of such a situation. In order to activate 4-hydroxytamoxifen to 4-hydroxytamoxifen quinone methide, which reacts with DNA, the 4-hydroxytamoxifen was incubated with horseradish peroxidase (HRP) and H_2O_2. As this activation step generates mutagenic oxidative DNA lesions, the HRP/H_2O_2 treatment without 4-hydroxytamoxifen was used as a control. In a *lacI* gene bacterial mutagenicity assay, 1 μM activated 4-hydroxytamoxifen induced mutations at a frequency ~16-fold higher than untreated control, whilst the HRP/H_2O_2 treatment itself induced mutations at a frequency ~7-fold higher than control. It was found that the mutagenicity of the HRP/H_2O_2 activated 4-hydroxytamoxifen was two orders of magnitude higher than that of α-acetoxytamoxifen in this system. Whilst the authors' conclusion that the adducts formed by 4-hydroxytamoxifen quinone methide are more mutagenic than the adducts formed by α-acetoxytamoxifen is justified, we would suggest that if our combined BPDE + UV results are indicative of a general enhancement of mutagenicity of combined exposures, then there is a possibility that some proportion of the higher mutagenicity of HRP/H_2O_2 activated 4-hydroxytamoxifen is due to the effects of the oxidative lesions enhancing the mutagenicity of the tamoxifen adducts, or *vice versa*.

If the presence of UV lesions and BPDE adducts in DNA does influence the mutagenicity of one or more of these adducts, it follows that mutations induced by agents that cause a range of adducts in DNA might demonstrate a similar effect. This would have implications for the conclusions drawn from experiments in which the mutagenicity of single DNA adducts are assessed in site-specific assays.

For the results we present here, we have not ruled out the possibility that the increase in GC→TA mutations is due to oxidative DNA damage induced by reactive oxygen species generated through an interaction of the BPDE adduct and UV radiation. We aim to investigate this and other questions that arise from these results in future experiments. For example, we aim to repeat the treatments of pSP189 in the reverse order (i.e. UV irradiation prior to BPDE adduction). This is unlikely to lead to enhanced single strand breaks, but will test whether the presence of UV and BPDE adducts on the same plasmid interact to increase mutation frequency. The results presented here demonstrate that UV-induced DNA damage and mutagenicity is enhanced by the presence of one class of bulky aromatic DNA adducts. This observation may be directly relevant to the enhanced toxicity of PAH by UV that has been observed in aquatic organisms, and may also be indicative of a general effect of synergistic mutagenicity by combined exposures of genotoxic agents. This possibility warrants further investigation.

Finally, the supF assay has been applied to a wide range of mutagenic treatments and of the 255 possible base substitution

mutations in the 85 base pair tRNA gene sequence, 245 have previously been reported (40). We note that in the experiments reported here we can add two more mutations to that list: a C→G transversion at site 142 (BPDE spectrum, Figure 2) and an A→C at site 157 (UVB spectrum, Figure 2).

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