STUDIES ON UV-INDUCED DNA DAMAGE AND REPAIR TO HUMAN DNA

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Doctor of Philosophy

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by

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ABSTRACT

The induction and repair of DNA damage has been shown to occur heterogeneously throughout the mammalian genome. As a consequence, analysis of these parameters at a global genome level may not reflect important gene-level events. Few techniques have been established to explore quantitatively gene-specific DNA damage and repair. Most of these are PCR-based assays and are relatively insensitive, relying on decreased PCR amplification arising from damage in template DNA. In this study, a semi-quantitative assay that combines specific immunocapture of damaged DNA by an antiserum specific for thymine dimers (IgG479), with PCR amplification of a 149bp fragment of the human H-*ras* proto-oncogene was established. Quantification of DNA damage was based upon proportionality between the amount of the PCR product and the initial amount of damage. Detection of thymine dimers was possible with nanogram amounts of genomic DNA and increased in a linear, dose-responsive manner. Using this assay, measurement of the induction of thymine dimers in the H-*ras* gene was shown to be proportional to the measurement of dimers induced in the global genome of UVC- and UVB-irradiated genomic DNA, as measured by gas chromatography-mass spectrometry and enzyme-linked immunosorbent assay. This result suggests that global damage assessments do indeed reflect gene-level events. However, the preferential repair of UVB-induced T<>T in the human H-*ras* proto-oncogene compared to the overall genome of human keratinocyte cells was detected. These findings demonstrate the suitability of this approach to the detection of UVR-induced DNA damage and repair at the level of individual genes.

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LIST OF ABBREVIATIONS

Abbreviation	Full name
$(NH_4)_2SO_4$	Ammonium sulphate
°C	Degree celsius
(6-4)PP	Pyrimidine-pyrimidone (6-4) photoproduct
8-oxodG	8-oxo-2'-deoxyguanosine
A	Adenine
*Bam*HI	*Bacillus amyloliquefaciens* H
BER	Base excision repair
BSA	Bovine serum albumin
BCC	Basal cell carcinoma
C	Cytosine
CC	Cytosine-cytosine dimers
cm	Centimeter
CPD	Cyclobutane pyrimidine dimer
CT	Cytosine-thymine dimer
dATTP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanosine triphosphate
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonucleic acidase

dNTP	Deoxynucleotide triphosphate
ds	Double-stranded
dsDNA	Double stranded DNA
dTTP	Deoxythymine triphosphate
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GC-MS	Gas chromatography-mass spectrometry
H_2SO_4	Sulphuric acid
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
hr	Hour
ICPCR	Immuno-coupled PCR
IgG	Immunoglubulin G
Kb	Kilobase
KCl	Potassium chloride
Kg	Kilogram
KJ	Kilojoule
LMPCR	Ligation-mediated PCR
M	Molarity (mol/L)
m^2	Meter square
MED	Minimal erythema dose
mg	Milligram

$MgCl_2$	Magnesium chloride
min	Minutes
mJ	Millijoules
ml	Milliliter
mm	Millimeter
mM	Millimolar
MM	Malignant melanoma
MMR	Mismatch repair
NaCl	Sodium chloride
NB-UVB	Narrow-band UVB
ng	Nanogram
NER	Nucleotide excision repair
NMSC	Non-melanoma Skin Cancer
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Poly(A)	Polyadenine
Poly(C)	Polycytosine
Poly(dC)	Polydeoxycytidylic acid
poly(dT)	Polydeoxythymidylic acid
Poly(G)	Polyguanine
Poly(T)	Polythymidylate
PUVA	Psoralen and ultraviolet A
QPCR	Quantitative PCR
ROS	Reactive oxygen species
SCC	Squamous cell carcinoma

SD	Standard deviation
Sec	Second
SEM	Standard error of mean
ss	Single stranded
ssDNA	Single stranded DNA
T	Thymine
T<>T	Cyclobutane thymine dimer
Taq	*Thermophilus aquaticus*
TC	Thymine-cytosine dimer
TCR	Transcription-coupled repair
TE	Tris-EDTA
Tg	Thymine glycol
Tris	Trizma base
Tris-HCl	Trizma hydrochloride
TT	Thymine-thymine dimer
UV	Ultraviolet light
UVA	Ultraviolet A (320-400nm)
UVB	Ultraviolet B (290-315nm)
UVC	Ultraviolet C (190-280nm)
UVC-poly(dT)	UVC-irradiated polythymidylic acid
UV-dsDNA	UV-irradiated double-stranded DNA
UV-poly(T)	UV-irradiated polythymidylate
UVR	Ultraviolet radiation
UVR-DNA	Ultraviolet irradiated DNA
UV-ssDNA	UV-irradiated ssDNA

V	Voltage
µg	Microgram
µl	Microliter

CHAPTER 1: INTRODUCTION

CHAPTER 1

INTRODUCTION

1.0 ULTRAVIOLET RADIATION

Ultraviolet radiation (UV) is part of the electromagnetic spectrum occurring between X-rays and visible light, comprising the wavelengths between 200 to 400nm, and is divided into three main regions of wavelengths. These are ultraviolet C (UVC, 200-280nm), ultraviolet B (UVB, 280-320nm) and ultraviolet A (UVA, 320-400nm) (Nataraj *et al.*, 1995) (Figure 1.1), each of which has different biological consequences after exposure to the human skin. Wavelengths lower than 290nm are heavily absorbed by molecular oxygen and ozone and do not reach the surface of the earth (Nataraj *et al.*, 1995), although some accidental exposure occurs from germicidal lamps. Thus, the longer wavelength UVB (295-320nm) and UVA (320-400nm) radiation, which constitute about 5% and 95%, respectively, of the solar spectrum are biologically relevant as they both reach the earth's surface in amounts sufficient to have important biological consequences from exposure of the skin (Freeman *et al.*, 1989).

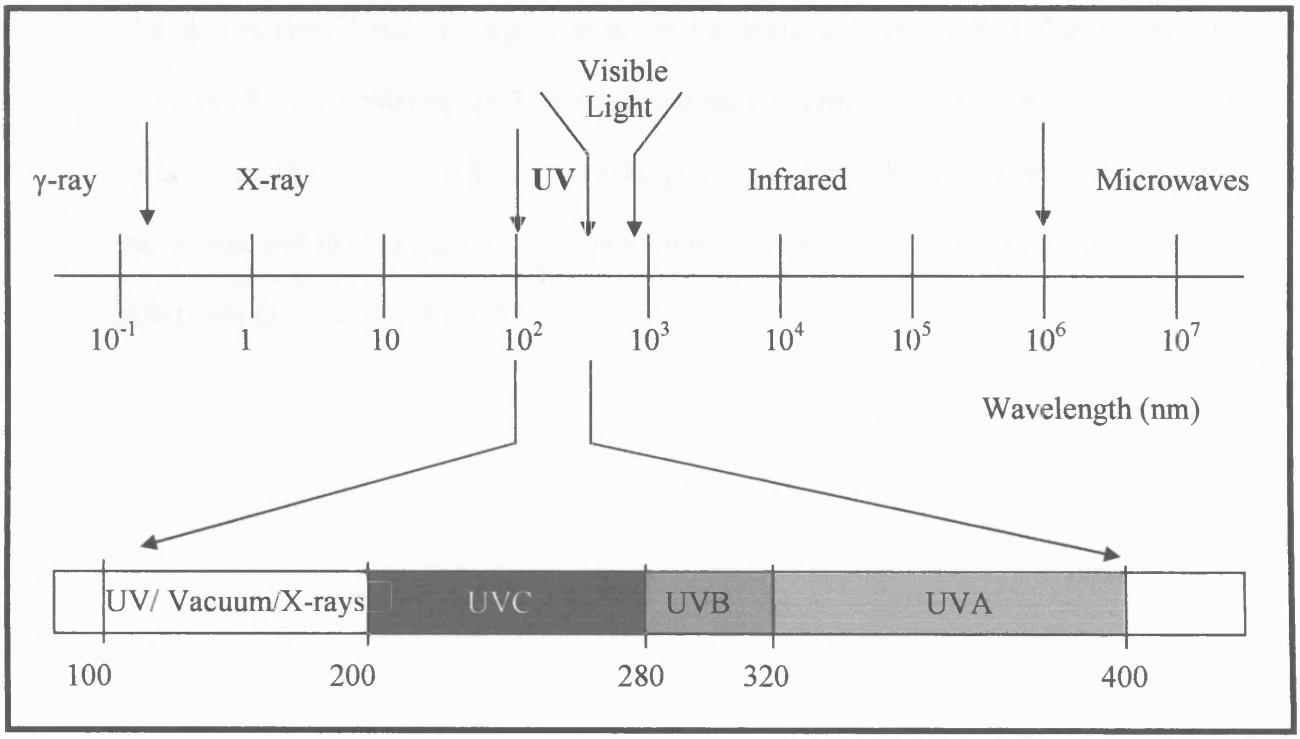


Figure 1.1: The ultraviolet (UV) component of the electromagnetic spectrum. Figure shows the division of electromagnetic spectrum and the three different sections of UV radiation (modified from Soehnge *et al.*, 1997).

1.1 THE SKIN

The skin is considered the largest organ of the body and has many different functions, including thermoregulation, protection, metabolic functions and sensation. Underneath the surface, the skin's multiple functions take place over three distinct layers: the epidermis, the dermis, and the hypodermis, each providing a distinct role in the overall function of the skin (Ross *et al.*, 2003) (Figure 1.2).

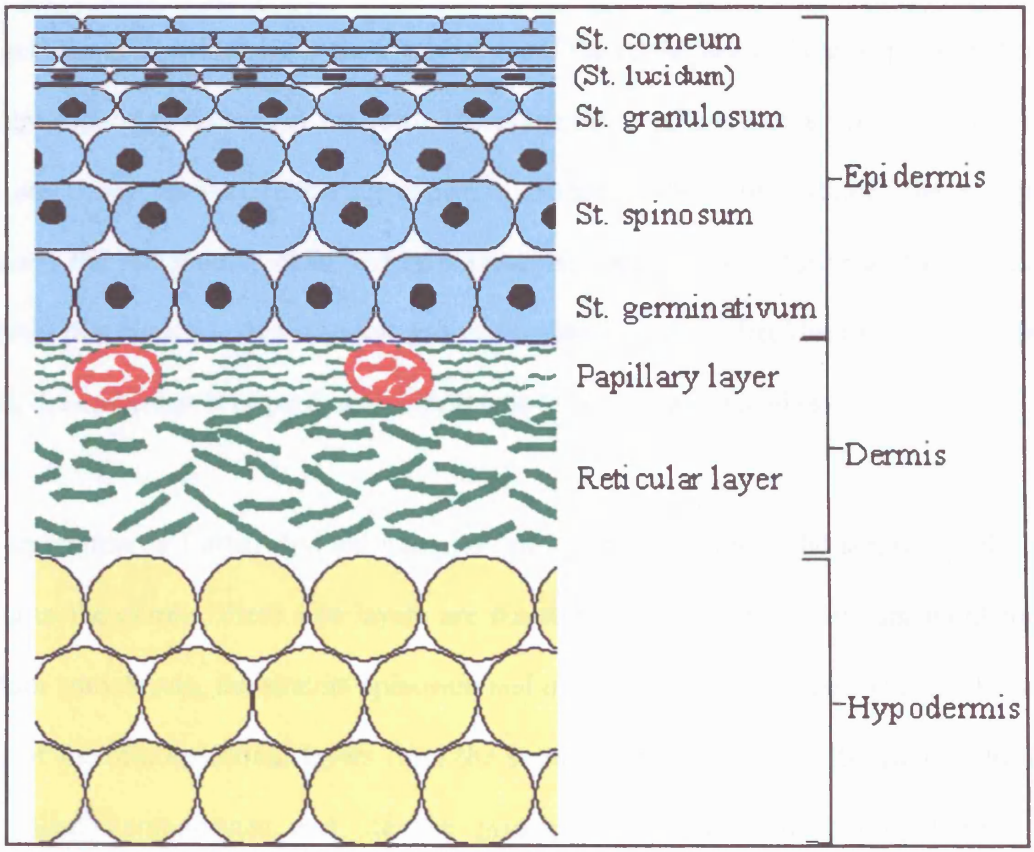


Figure 1.2: A schematic drawing of the layers of the skin. Figure shows the three distinct layers of the skin, epidermis, dermis and hypodermis, and the five sublayers of the epidermis (adapted from Ross *et al.*, 2003).

The epidermis is the most superficial layer of the skin and provides the first barrier of protection from the invasion of foreign substances into the body. It contains the Langerhans cells, which lie in the lower layers of the epidermis, and are responsible for the immunological protection of the skin. The Langerhans cells are sensitive to UV radiation and are easily harmed following exposure (Kripke, 1984). In addition, the epidermis contains the melanocyte cells and tyrokinase enzyme, which produce a pigment called melanin that gives the skin its color and pigmentation spots or freckles (Ross *et al.*, 2003). Thus, the epidermis is of particular importance to UV radiation studies.

The epidermis is further divided into five layers or strata. From the surface of the skin down to the dermis, these five layers are the stratum corneum, the stratum lucidum, the stratum granulosum, the stratum spinosum and the stratum germinativum (Figure 1.2). The cells of the four epidermal layers from the stratum germinativum to the stratum lucidum are called keratinocytes and are the principal cells found in the epidermis. The germinativum layer, also referred to as basal layer, is the living, growing part of the epidermis where the cells are reproduced by mitotic division. Following generation of cells from the basal layer, the cells move upward towards the stratum corneum pushing the cells above to the surface of the skin. As the cells move upwards, they undergo a progressive maturation called keratinisation, in which they are filled with keratin, a granular substance, lose their moisture and become flatten. Finally, the cells die and become part of the stratum corneum, which is the layer of dead epithelial cells and keratin that forms a protective barrier over the underlying living cells. The stratum lucidum is normally only well seen in thick epidermis and represents a transition from the stratum granulosum to the stratum corneum. In a young person, a cell requires approximately 28 days traveling from the

geminativum to the corneum layer of the skin whereas in an older person about 37 days are required by the cell to complete the same process (Ross *et al.*, 2003).

The second layer of the skin is much thicker than the epidermis and is called the dermis. The dermis lies below the epidermis and it consists of two connective tissues, the reticular layer, which is the deepest and made of dense irregular connective tissue, and the papillary layer that consists of loose connective tissue and lies adjacent to the epidermis. The dermis contains collagen and elastin protein fibers that give the skin its elasticity as well as a vast network of capillaries and blood vessels, which provide the skin's nutrition. The hypodermis, also called subcutaneous connective tissue, is the third skin layer and connects the skin with the muscle tissues. The hypodermis is the deepest of the three skin layers and is made of connective and fatty tissue. Hypodermis varies in thickness throughout the body depending on the part of the body being evaluated and the fat content of the individual (Ross *et al.*, 2003).

1.2 UV AND SKIN CANCER

Ultraviolet radiation is the major carcinogenic factor in sunlight and one of the most important physical agents that damage DNA (Brash, 1988; Kanjilal and Ananthaswamy, 1996). UV radiation results in altered keratinocyte differentiation by damaging their ability to proliferate leading thus, to their neoplastic transformation (Gloster and Brodland, 1996). The epidermal keratinocytes of the skin are the most susceptible to damage by exposure to UV radiation due to their localisation relative to the skin surface. Thus, most of the skin cancers in humans arise from the epidermis (Gloster and Brodland, 1996). Chronic exposure to UV radiation causes damage to the skin resulting in the formation of erythema, edema, hyperplasia, sunburn and photoaging (Young, 1990; Gloster and Brodland, 1996). The skin responds to sun exposure by tanning and thickening of the outer layer of the epidermis, which provides protection from further damage by UV radiation (Gloster and Brodland, 1996). In addition, UV radiation induces immunosuppression, mainly resulting from the induction of suppressor T cells, such as damaged Langerhans cells or inflammatory macrophages that enter the skin following UV exposure, leading eventually in the development and growth of tumours in the skin (Kripke, 1984; 1991). Furthermore, Ullrich (1995) suggested the release of soluble factors including IL-10, TNF-alpha, and IL-1alpha cytokines, which are secreted by keratinocytes after UVR-induced DNA damage, to be effective in the suppression of the immune system and prevention of T-cell mediated responses.

UV radiation is the most important, harmful and mutagenic component of the solar radiation spectrum due to its potential to damage DNA resulting in the formation of cutaneous malignancy in humans (Nataraj *et al.*, 1995; Black *et al.*, 1997). Studies using experimental animals have demonstrated that wavelengths in the UVB (295-320nm) region

of the sun's spectrum are the major cause of non-melanoma (NMSC) and melanoma forms of skin cancer (Forbes, 1981; deGruijl *et al.*, 1983). Indeed, although its low percentage (5%) in the solar spectrum, UVB radiation is considered to be the most important component of sunlight for the induction of skin malignancies in humans (Brash *et al.*, 1991; Kraemer, 1997). The most common types of cutaneous malignancy are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). The risk of NMSC is highest in people who sunburn easily and rarely tan (Gloster and Brodland, 1996). The incidence of NMSC is likely to increase further due to an increase in UVB radiation reaching the earth's surface, mainly caused by depletion of the ozone layer by human atmospheric pollutants, such as chlorofluorocarbons, (Crutzen, 1992; Kerr and McElroy, 1993) emphasising thus, the importance of increased cancer prevention and treatment efforts. BCC is the most frequent malignant skin tumour, occurring mostly in the elderly, and present at almost any sun-exposed sites, except palms and soles of feet (Kanjilal and Ananthaswamy, 1996) while SCC develops on sun-exposed, traumatised or chronically inflamed body sites (Gloster and Brodland, 1996). BCC and SCC are both keratinocyte-derived tumours, which show marked differences in biological behaviour (Kanjilal and Ananthaswamy, 1996). BCC is a slow-growing tumour of the skin keratinocyte and resemble the basal layer of the epidermis (Strom, 1996). Although BCC hardly ever metastasises, invasive growth may cause considerable local tissue destruction (Gloster and Brodland, 1996). BCC mainly occurs as a sporadic tumour but also in a hereditary form in nevoid basal cell carcinoma syndrome (NBCCS), an autosomal dominant disorder characterised by multiple, recurring BCC, general cancer susceptibility and developmental abnormalities (Gailani *et al.*, 1996; Wolter *et al.*, 1997).

In contrast, SCC is formed of more differentiated keratinocytes and tends to be a faster growing, locally invasive tumour with distinct metastatic potential (Strom, 1996). SCC may also develop from either pre-tumour precursor lesions such as actinic keratosis (Strom, 1996). Although these tumours can be readily distinguished at a clinical and microscopic level, studies to date have failed to identify consistent differences at a molecular level between these two biologically distinct keratinocyte-derived tumours (Andres *et al.*, 1994).

Malignant melanoma is a more serious type, with higher mortality, of skin cancer than the more common cancers, basal and squamous. In this malignancy, the cancer cells are found in melanocytes and account for most of the skin cancer deaths (Armstrong, 1988). Melanoma can metastasize quickly to other parts of the body through the lymph system or through the blood. Although melanoma can affect the skin on any part of the body, the commonest place in women is on the legs, while in men it is more common on the back (Armstrong, 1988). It has also been found that melanoma incidence correlates with blistering sunburn in childhood or adolescence (Fitzpatrick and Sober, 1985). Melanoma sites are also associated with the dysplastic melanocytic nevi, which are distinctive melanocyte moles that most likely represent both a marker for those patients at an increased risk for development of melanoma and a precursor lesion to melanoma (Fitzpatrick and Sober, 1985).

1.3 DIRECT UV-MEDIATED DAMAGE TO DNA

The continuity of species from one generation to the next is attributed to the stability of DNA. In spite of its essential role as the carrier of genetic information, DNA is not an inert structure. The DNA of living cells is susceptible to potentially mutagenic threats from both

endogenous sources, such as hydrolysis and oxidation, and exogenous sources, including ultraviolet and ionising radiation, as well as a wide variety of chemical carcinogens (Friedberg *et al.*, 1995). Although all the primary components of DNA (sugars, bases and phosphodiester linkages) are subject to damage, the nitrogenous bases are more important, since these are the informational elements of the genetic blueprint encoded in DNA (Friedberg *et al.*, 1995).

The response of human cells to the damaging effects of UV radiation has been studied using the model mutagen 254nm UVC, although it is not biologically relevant for sun exposure, as previously described. The high efficiency of photochemical reactions within DNA occurring at this wavelength occur because the maximal wavelength of UVC is very close to the absorption maximum of pyrimidine and purine bases in DNA (Ravanat *et al.*, 2001). Indeed, DNA absorbs radiation maximally from 245 to 290nm (Tornaletti and Pfeifer, 1996) resulting in the formation of mutagenic photolesions in DNA between adjacent pyrimidines in the form of dimers (Friedberg *et al.*, 1995). These dimers, formed more efficiently by UVC radiation, are of two main types: cyclobutane pyrimidine dimer (CPD) and pyrimidine-pyrimidone (6-4) photoproduct [6-4(PP)] (Cadet and Vigny, 1990). CPD arises from the formation of a cyclobutane ring between the 5-carbon atoms and the 6-carbon atoms of two adjacent pyrimidines. The double bonds become saturated to produce a four-membered ring known as a cyclobutane ring (Cadet and Vigny, 1990). Thymine-thymine (T-T) dimers are formed preferentially, but cytosine-cytosine (C-C) and cytosine-thymine (C-T) dimers can also occur (Douki and Cadet, 2001). CPDs can exist in diastereoisomers generated from thymidine in the form of *cis-syn*, *trans-syn*, *trans-anti* and *cis-anti* (Douki *et al.*, 2003). CPDs formed in DNA exist predominantly in the *cis-syn* form

with the cyclobutane thymine-thymine pyrimidine dimer (c-s T<>T) found to exist in higher excess in UV-irradiated DNA (Douki *et al.*, 2003) (Figure 1.3).

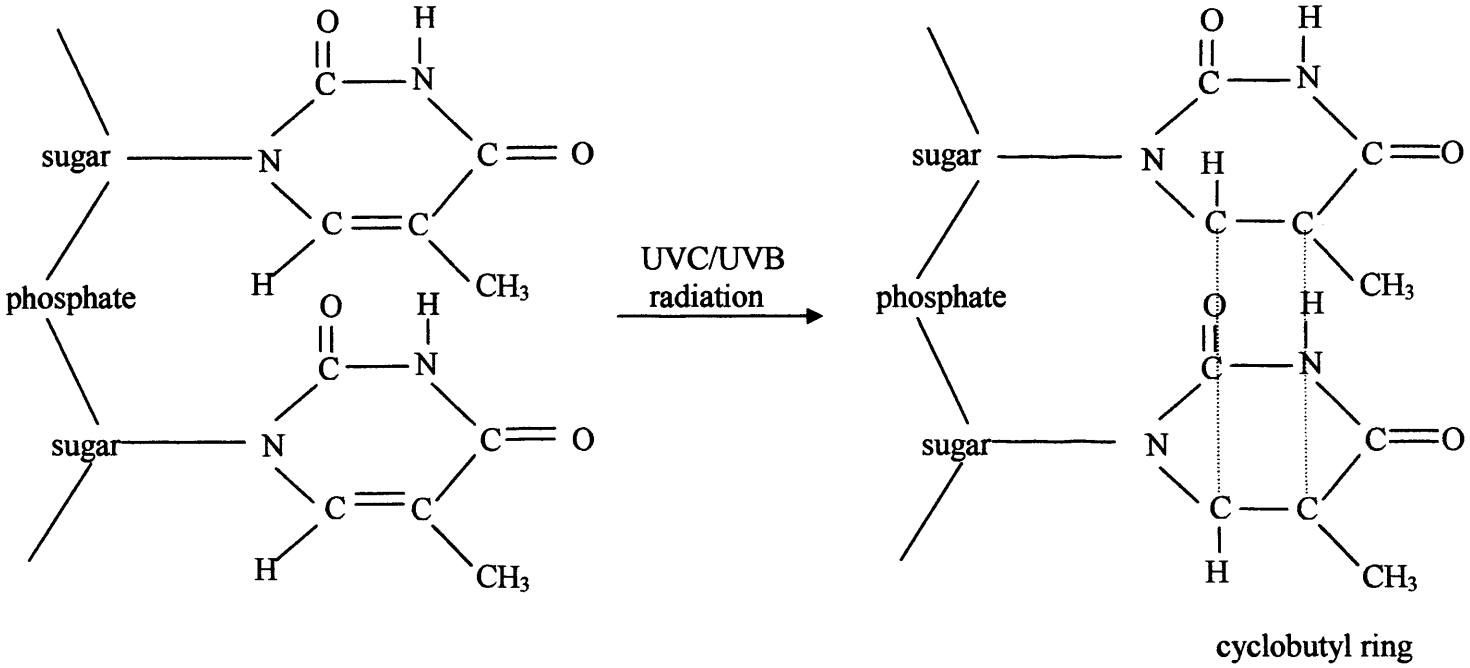
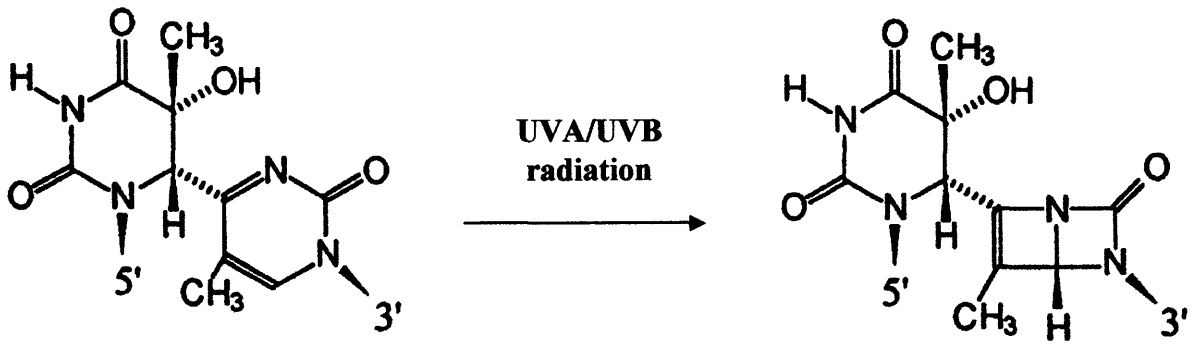


Figure 1.3: Structure of a *cis-syn* cyclobutane thymine dimer. Following UVC or UVB radiation adjacent thymine residues in a DNA strand are joined by formation of two bonds shown in red colour (modified from Friedberg *et al.*, 1995).

The second type of dimer induced by UV radiation involves the joining of the 5' C-4 position and the 3' C-6 position of two adjacent pyrimidine residues to form a 6-4 lesion, called 6-4(PP) (Mitchell and Nairn, 1989). The latter lesions can be converted into their Dewar valence isomers (Dewar PP) formed *via* photoisomerisation at wavelengths around 320nm (Taylor *et al.*, 1990) (Figure 1.4). The (6-4) photoproducts can exist as TT, TC and CT (6-4)PPs along with their Dewar valence isomers (Douki *et al.*, 2000) but (6-4)PPs are most often formed between CC and TC residues (Tornaletti and Pfeifer, 1996). CPDs and (6-4)PPs can be readily formed by UVB radiation through direct absorption of UVB photons by DNA, although the yield is much lower. Indeed, the rate of CPD formation by UVB radiation was found to be 100 times lower than UVC (Perdiz *et al.*, 2000). CPD and (6-4) PP are formed in a 3:1 ratio, respectively (Tornaletti and Pfeifer, 1996), although their yield and distribution depend on the DNA sequence, the local DNA structure and the chromatin environment (Pfeifer, 1997; Thoma, 1999). The chromatin environment particularly has been shown to affect UV-induced damage formation distributions in nucleosomes isolated from UV-irradiated cells (Gale *et al.*, 1987; Pehrson, 1995).



(6-4)PP **Dewar**

Figure 1.4: Structure of pyrimidine-pyrimidone (6-4) photoproduct [6-4(PP)] and its Dewar valence isomer. Following exposure to UVA or UVB radiation, (6-4)PPs can be converted into their Dewar valence isomers *via* photoisomerisation (modified from Cadet and Vigny, 1990).

However, although UVA radiation is weakly absorbed by DNA relative to UVC or UVB (Sutherland and Griffin, 1981), it has been reported to give rise to CPDs (Kvam and Tyrrell, 1997; Perdiz *et al.*, 2000). Recent studies have shown the induction of CPDs, especially at TT sites, in Chinese hamster ovary cells following irradiation with UVA (Douki *et al.*, 2003; Rochette *et al.*, 2003), *via* a photosensitised triplet energy transfer from an excited, yet unidentified, chromophore to thymine. The preferential formation of UVA-induced CPDs *via* triplet energy transfer in the presence of chromophores, such as aromatic ketones, has been previously reported *in vitro* (Charlier and Helene, 1972; Moysan *et al.*, 1991), suggesting a role of CPDs in UVA solar mutagenesis.

Although both CPDs and (6-4)PPs photolesions are potentially mutagenic (Brash *et al.*, 1991), CPD is considered the most mutagenic lesion based on its abundance, slow repair and distinct mutagenicity (Pfeifer, 1997; Yoon *et al.*, 2000). CPD is believed to be the major contributor to mutations in mammals, while the [(6-4) PP] being more important in *Escherichia coli* cells (Brash, 1988; Tornaletti and Pfeifer, 1996). In addition, UVR-induced pyrimidine dimers may influence important cellular functions, including transcription, replication and DNA repair (Friedberg *et al.*, 1995; Tommasi *et al.*, 1997). It has been shown that the presence of T<>T in promoter sequences can strongly inhibit transcription factor binding (Tommasi *et al.*, 1996) and block transcription elongation when present on the transcribed strand (Donahue *et al.*, 1994). If not repaired, the UVR-induced photolesions can lead to permanent mutations in the form of C→T transitions and CC→TT tandem transitions at dipyrimidine sequences, known as UV 'signature' mutations, as they specifically induced by UV light (Ziegler *et al.*, 1993) resulting eventually in the development of cancer. In addition, a C-T mutation occurs when an A

residue is placed opposite a C residue by default during the formation of a (6-4)PP between a pyrimidine and a C residue (Kanjilal and Ananthaswamy, 1996).

In addition to CPDs and (6-4)PPs, other photoproducts are also produced by UVC and UVB radiation, including pyrimidine monoadducts, purine dimers, 8,8-adenine dehydrodimer and a photoproduct at TA sequences (Bose and Davies, 1984; Gasparro and Fresco, 1986; Tornaletti and Pfeifer, 1996) but at much lower frequencies.

1.4 INDIRECT UV-MEDIATED DNA DAMAGE

Visible light and near ultraviolet radiation (320-400nm) (UVA) have been associated with genotoxic, cytotoxic, mutagenic and carcinogenic properties (Peak and Peak, 1989; Drobetsky *et al.*, 1995). UVA is the predominant component of solar UV radiation to which everybody is exposed. However, it appears weakly carcinogenic as it is poorly absorbed by DNA (Sutherland and Griffin, 1981). The DNA-damaging, carcinogenic and mutagenic properties of UVA radiation result from oxidative DNA base modifications, which are formed following an indirect reaction *via* the excitation of a cellular photosensitiser, leading to the generation of oxygen species mainly singlet oxygen (Peak and Peak, 1990; Ravanat *et al.*, 2001). Kvam and Tyrrell (1997) have determined the wavelength dependent yield of 8-oxodG, a non-dimer oxidative intermediate, to be in the range of 312 to 365nm after exposure of non-growing human skin fibroblasts to monochromatic UVB radiation, suggesting the UVA-induced singlet oxygen as responsible in the induction of the 8-oxodG. High levels of 8-oxodG have been shown in epidermal cells of hairless mice after chronic or single exposure to UVB radiation (Beehler *et al.*, 1992; Hattori *et al.*, 1997) and in normal human epidermis after a single dose of ultraviolet

radiation (Ahmed *et al.*, 1999) suggesting a role of 8-oxodG in sunlight-induced skin carcinogenesis.

1.5 OXYGEN FREE RADICALS, OXIDATIVE STRESS AND REACTIVE OXYGEN SPECIES

Oxygen free radicals are a natural byproduct of respiration. Electrons formed during the oxidation of glucose are passed along the electron transport chain, a series of electron-accepting molecules embedded in the mitochondrial membrane. Protons created during electron transfer ultimately are used to drive the synthesis of ATP. In the final step of transfer, electrons are combined with oxygen and protons to produce water (Halliwell, 1993).

Reactive oxygen species (ROS) are oxygen containing molecules like hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), which are non-radicals (paired electrons) and radicals like the hydroxyl radical ($OH^{\bullet}$) and the superoxide anion ($O_2^{-\bullet}$) and oxides of nitrogen ($NO^{\bullet}$, $NOO^{\bullet}$) (Halliwell, 1993).

The steady-state formation of pro-oxidants in cells and organs is balanced by a similar rate of their consumption by antioxidants that are enzymatic and/or nonenzymatic. "Oxidative stress" results from imbalance in this pro-oxidant-antioxidant equilibrium in favor of the pro-oxidants (Halliwell and Gutteridge 1995). A disturbance in this balance can occur either by a reduction in antioxidant or an increase in the levels of ROS (Halliwell and Gutteridge 1995). However, a proportion of ROS escaping the antioxidant defenses result,

even in the absence of oxidative stress, in a background level of oxidative DNA damage (Cooke *et al.*, 2000a).

1.6 MECHANISMS OF FORMATION OF REACTIVE OXYGEN SPECIES

Photodynamic reactions are potential mechanisms by which ultraviolet radiation damages living cells (Ito, 1983). DNA is one of the key targets for UVR-induced damage in a variety of organisms such as bacteria (Peak and Peak, 1982; Peak *et al.*, 1984), plants (Quaite *et al.*, 1992), animal and humans (Stein *et al.*, 1989; Kripke *et al.*, 1992). All biological organisms contain UV-absorbing agents including nucleic acids and proteins, flavins, porphyrins, quinones (Ito, 1983) as well as NADH and NADPH dehydrogenase (Cunningham *et al.*, 1985), which act as chromophores leading to the formation of singlet oxygen or free radicals. Superoxide anion is generated continuously by several cellular processes including the microsomal and mitochondrial electron transport systems. Cells of the immune system have a special role in production of superoxide anion since they contain a membrane-bound enzyme complex, the NADPH oxidase that reduces oxygen with NADPH to produce copious amounts of superoxide anion (Cunningham *et al.*, 1985).

ROS are produced during normal cellular function and are generated as by-products of cellular metabolism primarily in the mitochondria (Halliwell, 1993). The rate of formation depends on the amount of oxygen flowing through the mitochondria at any given time. However, faulty electron transfer at any point in the electron transport chain, results in an electron being accepted by atomic oxygen (O_2). The superoxide free radical created has a single unpaired electron (designated by the "dot" in the chemical formula), which seeks to react with an electron source to make a stable electron pair. Under physiological conditions, electrons "leak" from the electron transport chain, converting oxygen

molecules into superoxide (Evans *et al.*, 1997a). Hydrogen peroxide readily diffuses out of the mitochondria, and its level in the cytoplasm may provide the cell a means to monitor the efficiency of respiration (Evans *et al.*, 1997a). Hydrogen peroxide is unique in that it can be converted to the highly damaging hydroxyl radical or be catalyzed and excreted harmlessly as water. If H_2O_2 is not converted into water, singlet oxygen ($^1O_2$) is formed. Singlet oxygen is not a free radical, but can be formed during radical reactions and also cause further reactions. Singlet oxygen can act as a catalyst by transferring the energy to a new molecule, which in turn can interact with other molecules leading to the formation of a new free radical (Halliwell, 1991). Autoxidation of semiquinones can also lead to the activation of oxygen, producing superoxide anion radical as shown it the reaction below (Guillén *et al.*, 2000).

$$Q^{-\bullet} + O_2 \rightarrow Q + O_2^{-\bullet}$$

Hydrogen peroxide (H_2O_2) can also be generated by the same sources that produce superoxide anion since two molecules of superoxide anion dismute to hydrogen peroxide and oxygen readily in a reaction catalyzed by the enzyme superoxide dismutase. Dismutases are metal-containing enzymes and form a crucial part of the cellular antioxidant defense mechanism (Halliwell, 1991).

$$2O_2^{-\bullet} + 2H^+ \xrightarrow{\text{superoxide dismutase}} H_2O_2 + O_2 \qquad [1]$$

In addition, superoxide anion and hydrogen peroxide can be consumed by interaction with transition metals, such as copper or iron *via* the Haber-Weiss reaction (Halliwell, 1991). These reactions are significant as the substrates are found within the body and could easily interact.

HABER-WEISS REACTION

$$O_2^{-\bullet} + H_2O_2 \rightarrow O_2 + HO^- + HO^\bullet \quad [2]$$

If the transition metal is ferrous iron (Fe^{2+}) the process is known as the Fenton reaction where the addition of one electron to the oxygen-oxygen bond of hydrogen peroxide (H_2O_2) results in the formation of hydroxyl radical ($OH^{-\bullet}$) and ferric ion (Fe^{3+}) (Halliwell and Gutteridge, 1992).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^\bullet \quad [3]$$

Fenton Reaction

In biological systems the availability of ferrous ions limits the rate of reaction, but the recycling of iron from the ferric to the ferrous form by a reducing agent, such as superoxide, can maintain an ongoing Fenton reaction leading to the generation of hydroxyl radical.

$$Fe^{3+} + O_2^{-\bullet} \rightarrow Fe^{2+} + O_2 \quad [4]$$

Therefore, in the presence of trace amounts of iron, the reaction of superoxide and hydrogen peroxide will form the destructive hydroxyl radical and initiate the oxidation of organic substrates. ROS can also be generated by the activity of phagocytic cells like neutrophils and macrophages induced at sites of inflammation (Halliwell and Gutteridge 1985).

1.7 CONSEQUENCES OF OXIDATIVE DNA DAMAGE

Reactive oxygen species (ROS) have been shown to be involved in various processes, including activation of cytoplasmic and nuclear signal transduction pathways, alteration of

DNA polymerase activity, modulation of gene expression and protein production (Wiseman *et al.*, 1995). Hydroxyl radicals react with all types of biologically important molecules, such as nucleic acids, proteins, sugars, and lipids, resulting in the generation of DNA-protein crosslinks that interfere with chromatin unfolding, DNA repair, replication, and transcription, single- and double-stand breaks in DNA and sugar modifications (Peak *et al.*, 1987; Breen and Murphy, 1995; Altman *et al.*, 1995; Cadet *et al.*, 1999). In addition, ROS can structurally modify lipids *via* lipid peroxidation and, as a consequence, to the formation of reactive aldehydes (Marnett, 1999; Nair *et al.*, 1999). As a result of their ability to modify cellular biomolecules, ROS are involved in a variety of pathological conditions including inflammation (Frenkel *et al.*, 1993), carcinogenesis (Kensler and Taffe, 1986; Floyd, 1990), and may act as promoters and initiators of carcinogenesis (Guyton and Kensler, 1993; Simic, 1994), aging (Bunker, 1992; Ames *et al.*, 1993; Barnett and King, 1995) and autoimmunity (Lunec *et al.*, 1994; Cooke *et al.*, 1997).

Of the more than 70 plus different oxidative DNA lesions the guanine-derived lesion 8-oxo-guanine (8-oxoG), which is also known as 8-oxo-7,8-dihydroguanine, and its corresponding deoxynucleoside 8-oxo-2'-deoxyguanosine (8-oxodG) appear to be the most abundant in DNA and biologic fluids, such as urine and serum, and the most mutagenic (Kasai, 1997; Poulsen *et al.*, 1998a; Weimann *et al.*, 2002). 8-oxodG is the most extensively analysed of all the oxidative DNA damage products, because it is the most easily measured (Marnett, 2000). 8-oxodG has become a popular marker for evaluating oxidative DNA damage in human skin, because it induces the biologically significant GC to TA transversions during DNA replication after mispairing with adenine residues (Shibutani *et al.*, 1991; Musarrat *et al.*, 1996). 8-oxodG is a block to transcription in human cells, if not repaired (Le Page *et al.*, 2000). Its mutagenic potential is further supported by

the presence of GC to TA transversions in the *p*53 tumour suppressor gene and the *ras* oncogenes in UVB-induced skin cancers in mice (Nishigori *et al.*, 1994) and in human non-melanoma skin cancer (van der Schroeff *et al.*, 1990). GC to CG transversions by mispairing of guanine opposite 8-oxoG has also been reported (Ames and Gold, 1991).

Furthermore, ROS are involved in the pathogenesis of chronic inflammatory skin diseases, such as atopic dermatitis (Tsuboi *et al.*, 1998) and psoriasis (Nagata *et al.*, 1995). 8-oxodG is one of the products which are excreted into urine as a result of oxidative damage to DNA and can be detected as a nucleoside derivative (Simic, 1992). The measurement of urinary oxidative DNA lesions is of great importance as they can be used as biological markers of oxidative damage to DNA and disease, particularly cancer (Tagesson *et al.*, 1995). Indeed, it has been shown that high urinary levels of 8-oxodG was the possible cause of the higher incidence of malignancy in patients with cystic fibrosis (Brown *et al.*, 1995). Furthermore, Simic (1992) suggested that the level of a single biomarker as 8-oxodG can be used as a relative measure of total DNA damage by hydroxyl radicals. Measurement of 8-oxodG in urine has also been shown to provide information regarding the intra- and inter-individual variability of excretion of this oxidative DNA lesion in relation to smoking, age, body weight, sex and body mass (Pilger *et al.*, 2001).

In addition to modifying DNA, oxygen radicals can react with polyunsaturated fatty acid residues in phospholipids, by abstracting a hydrogen atom from a reactive group and creating a lipid radical ($L^{\bullet}$). This lipid radical reacts with molecular oxygen to form lipid peroxyl radical ($LOO^{\bullet}$), which will subsequently initiate a new free radical attack via the extraction of a new hydrogen atom from a neighboring polyunsaturated lipid. This process

results in the formation of a plethora of products such as lipid hydroperoxides (LOOH) (Esterbauer, 1985). One of the most abundant products of lipid peroxidation is malondialdehyde (MDA), which also is generated as a side-product of prostaglandin biosynthesis and reacts with DNA to form mutagenic and carcinogenic adducts such as malondialdehyde-guanine (M_1G) in mammalian cells (Fang *et al.*, 1996; Wang *et al.*, 1996) and in *Escherichia coli* (Fink *et al.*, 1997). DNA adduction by MDA correlates to alterations in cell cycle control and gene expression in cultured cells (Ji *et al.*, 1998). Lipid peroxidation appears thus, to be a major source of endogenous DNA damage in humans that may contribute significantly to cancer and other genetic diseases.

In addition to 8-oxodG, other mutagenic lesions including *cis* thymine glycol (Tg), 8-oxo-adenine (8-oxoA) and its deoxynucleoside 8-oxo-2'-deoxyadenosine as well as the deaminated cytosine glycol products, including uracil glycol, 5-hydroxymethylurine, 6-hydroxy-5,6-dihydrocytosine and 5-hydroxyuracil (5-OHUra) (Dizdaroglu, 1993; Wang *et al.*, 1998) are produced as a result of oxidative damage to DNA (Figure 1.5).

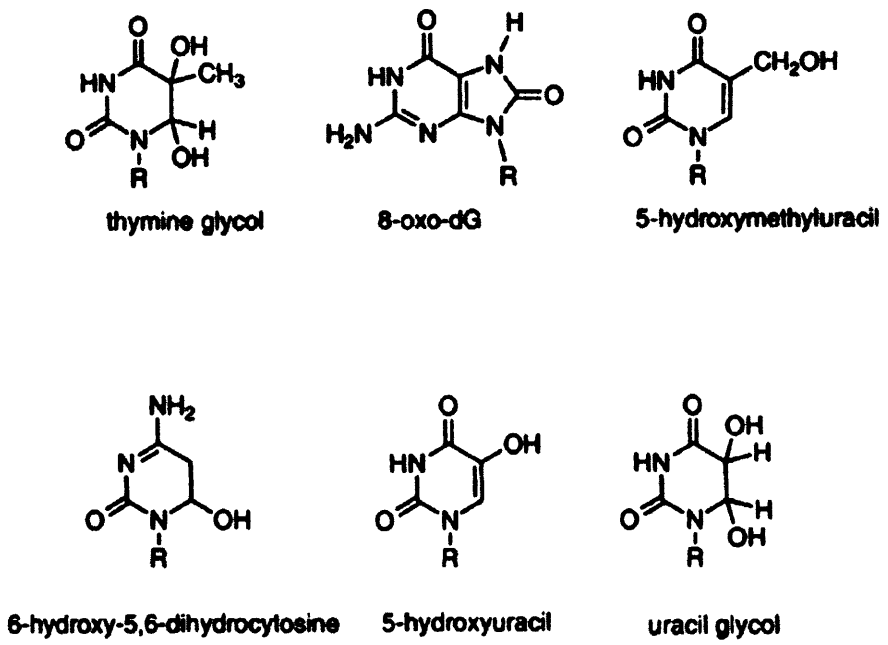


Figure 1.5: Structure of some major oxidative-induced DNA lesions.

(adapted from Marnett, 2000).

1.8 MECHANISMS OF UVR-INDUCED CARCINOGENESIS

Clinical, experimental and epidemiological evidence have shown that exposure to UV light is the major determinant in the development of skin cancer due to its potential to damage DNA leading thus, to the generation of potentially mutagenic lesions in the DNA sequence and eventually the development of cancer. Carcinogenesis by UV radiation is a complex series of events that involves initiation, promotion and progression (Forbes, 1981). It is a process characterised by the accumulation of multiple genetic as well as epigenetic changes, and by the selection for genetically unstable cells that evade the physiological controls of cell proliferation, apoptosis, cell differentiation and cell mortality (MacPhee, 1998; Rajewsky *et al.*, 2000). Genetic events result in changes in DNA sequence whereas epigenetic process alters the expression of the genetic information at the transcriptional, translational or post-translational level (Jones and Buckley, 1990; Jones and Laird, 1999). The genetic alteration mechanism has been supported by the presence of many endogenous and exogenous chemicals able to cause changes in the DNA sequence (Barrett, 1993). Some cancer researchers observed that embryogenesis and differentiation, which are characterised by specific patterns of gene expression in specific tissues and organs, proceed without any alterations in DNA sequences due to an epigenetic change (MacPhee, 1998). DNA methylation is considered to be the major cause of epigenetic change (Jones and Buckley, 1990) due to its inheritance even after DNA replication by maintenance methylation. DNA methyltransferase is known to be recruited to the replication foci (Araujo *et al.*, 1998), and it is suggested that maintenance of CpG sites takes place simultaneously with DNA replication.

Carcinogenesis can also occur by overactivation of growth enhancing genes (oncogenes) and the inactivation of growth inhibitory genes (tumor suppressor genes) resulting in the

progression stage of cancer development (Balmain *et al.*, 1988; Fearon and Vogelstein, 1990). Activation of oncogenes requires a change in only one copy of the gene compared to tumour suppressor genes where inactivation of both copies of the gene is required to have an effect (Knudson, 1985; Balmain *et al.*, 1988). The distinction between the terms proto-oncogene and oncogene relates to the activity of the protein product of the gene. A proto-oncogene is a gene whose protein product has the capacity to induce cellular transformation given it sustains some genetic insult while an oncogene is a gene that has sustained some genetic damage and, therefore, produces a protein capable of cellular transformation (Balmain and Brown, 1988). Proto-oncogenes act to control cell proliferation and differentiation of normal embryonic and adult tissues and are divided into three groups, growth factors and growth receptors, signal transduction proteins and nuclear factors (Kanjilal and Ananthaswamy, 1996). However, exposure of cells to carcinogenic agents may activate these normal genes by causing them to produce higher levels of their normal gene product or by inducing structurally aberrant gene products (Balmain *et al.*, 1988). Mutations in DNA may act as initiating events and they remain dormant for a long period of time until exposure to a promoting agent, such as UV radiation or chemical, takes place (Ananthaswamy and Pierceall, 1990). Several genes have been extensively studied to have important roles in skin carcinogenesis, including the tumor suppressor gene *p*53, the *ras* oncogenes as well as the *patched* (*PTCH*) gene (Gailani *et al.*, 1996; Wolter *et al.*, 1997).

The *ras* gene family is a eukaryotic gene family and consists of three functional proto-oncogenes Harvey *ras* (H-*ras*), Kirsten *ras* (K-*ras*), neuroblastoma-derived *ras* (N-*ras*), which encode highly similar (70% sequence homology) 21kDa guanosine triphosphate (GTP)-binding proteins located on the inner surface of the cell membrane (Barbacid, 1987;

McCormick, 1989). These proteins participate in signal transduction from the cell surface to the nucleus and in growth control through intrinsic GTPase activities (Barbacid, 1987). H-*ras* proto-oncogene, for example, is involved in a variety of cellular reactions, including transformation of established fibroblasts to a malignant state, mesoderm induction in *Xenopus*, and promotion of neurite outgrowth in normal neurons (Capon *et al.*, 1983). Several investigators have shown that oncogenes can operate in concert with other oncogenes or genetic changes to produce the transformed state in human cancer. *Ras* oncogenes, for example, can cooperate with *myc* genes to transform primary or secondary rat embryo fibroblasts when introduced together by DNA-mediated gene transfer (Spandidos and Lang, 1989).

Ras genes acquire oncogenic activity as a result of point mutations in codons 12, 13 and 61 of the three *ras* proto-oncogenes H-*ras*, K-*ras* and N-*ras* respectively producing single amino-acid substitutions, which alter the active site of the protein and its ability to bind or hydrolyze GTP (Bos, 1989). Activation of *ras* genes by mutation is very common in human cancers, and it may occur either early in cancer development or during tumor progression (Barbacid, 1987; Bos, 1989). Detection of single nucleotide substitution mutations in K-*ras* gene, for example, may be of diagnostic significance as an early tumor marker for pancreatic and colon cancers (Bos, 1989). In addition, mutations in the H-*ras* gene may be useful in detection and monitoring of patients with bladder cancers (Reddy, 1983; Capon *et al.*, 1983). Besides point mutations, cellular proto-oncogenes can be activated by chromosomal translocations, which involve the transfer of a gene from its normal site to an alternative site, such as a different chromosome, resulting in altered gene transcription, suggesting that may be a direct link between exposure to agents that damage DNA and genetic change leading to cancer (Zarbl *et al.*, 1985). Mutations of the *ras* genes

have also been reported in human skin cancers (Suarez *et al.*, 1989; Pierceall *et al.*, 1991). *Ras* gene mutations occur at pyrimidine rich sequences, suggesting that these sites are targets for UVR-induced DNA damage that leads to mutation and transformation (Sutherland *et al.*, 1980; Corominas *et al.*, 1989).

The tumour suppressor *p*53 gene holds the most prominent place in the field of cancer research. The *p*53 gene codes for a DNA-binding protein, which is a central element in fundamental cellular processes, including gene transcription, repair of DNA damage, control of the cell cycle, genomic stability, chromosomal aggregation and apoptosis (Harris, 1996). Mutations of the *p*53 tumor suppressor gene are found in about 50% of all human cancers (Greenblatt *et al.*, 1994). *P*53 mutations are mostly missense type (about 80%), where the encoded protein contains an amino acid substitution that alters *p*53 conformation and sequence-specific transactivation activity, resulting in correlations between distinct mutants and functional changes (Hollstein *et al.*, 1991; Greenblatt *et al.*, 1994). In addition, the missense mutations that alter *p*53 function are distributed over a large region of the molecule, especially in the hydrophobic midportion of the protein (Hollstein *et al.*, 1991; Greenblatt *et al.*, 1994). Other mutations, such as deletions and insertions, are also found in the *p*53 gene but with a lower frequency compared with that of other tumor suppressor genes, such as *APC, BRCA1* and *ATM* (Hollstein *et al.*, 1991). Mutations in the *p*53 tumor suppressor gene have been found in a large percentage of skin cancers, most being located in exons 5 through 9, which contain conserved sequence blocks (Tornaletti and Pfeifer, 1994). Mutations of the *p*53 gene are found in about 50% of UVR-induced skin cancers and are C→T transitions and CC→TT double transitions at dipyrimidine sequences, which are specifically induced by UV-light and known as UV 'signature' mutations (Tornaletti and Pfeifer, 1994; Brash *et al.*, 1991). *P*53 mutations are

associated with the high incidence of BCC and SCC in human skin cancer (Pierceall *et al.*, 1992; Ziegler *et al.*, 1993) as well as in skin cancers from patients with XP (Dumaz *et al.*, 1993; Sato *et al.*, 1993). This mutational specificity makes the *p*53 tumor suppressor gene an excellent model system to study possible correlations between the frequency of UVR-induced photoproducts at specific sequence locations and the distribution of mutations along the same sequences (Brash *et al.*, 1991; Tornaletti *et al.*, 1993).

The *p*53 tumor suppressor gene and the *ras* oncogens have been extensively studied, as they are unique models for studying the process of tumour induction and progression. However, there are also other important genes involved in carcinogenesis. One of these is the *patched* (*PTCH*) gene, which can act as a tumour suppressor in humans (Kanjilal and Ananthaswamy, 1996). The *PTCH* gene, which was originally identified in the fruit fly Drosophila, encodes a cell-surface protein that functions both in development and tumorigenesis through its ability to regulate a number of intercellular signalling molecules (Gailani *et al.*, 1996; Wolter *et al.*, 1997). Genetic and functional studies demonstrate that *PTCH* gene is part of the hedgehog signalling cascade, an evolutionary conserved pathway that regulates the expression of a number of downstream genes. Scientists have recently found BCC, the most common type of skin cancers in humans, results from mutations in both copies of a human gene that is similar to *PTCH* (Gailani *et al.*, 1996; Wolter *et al.*, 1997). NBCCS is a rare autosomal dominant disorder characterized by multiple BCCs and studies of NBCCS patients have shown to have both genomic and sporadic mutations in the *PTCH* gene, suggesting that these mutations are the major cause of this disease (Kress *et al.*, 1992; Gailani *et al.*, 1996; Wolter *et al.*, 1997). The involvement of *PTCH* gene in the inhibition of gene expression and the occurrence of frequent allelic deletions of *PTCH* in BCC support a tumor suppressor function of this gene (Kanjilal and Ananthaswamy,

1996). In addition, its role in the regulation of gene families known to be involved in cell signalling and intercellular communication provides a possible mechanism of tumor suppression (Gailani *et al.*, 1996; Wolter *et al.*, 1997).

1.9 PROTECTIVE MECHANISMS AGAINST DIRECT DAMAGE TO DNA

Protection from direct absorption of UV light could be achieved by less exposure to sunlight. Protective clothing appears to be a good blocker to UV light (Robson and Diffey, 1990; Stanford *et al.*, 1995). In addition, the use of hats is of great importance, since they cover the head and neck, which gets almost continual sun exposure, even in the winter. Study of multiple skin cancers suggested that hats may help to decrease the risk (Diffey and Cheeseman, 1992).

Tanning is essentially the skin's way of increasing its defense against the damaging effects of UVR radiation, as it protects against sun damage principally by increasing the melanin content of the epidermis. However, recent evidence suggests that tanning only occurs after DNA damage has already occurred (Eller *et al.*, 1994). Essentially, DNA damage is the trigger for the tanning response, meaning that tanning begins after some damage has already occurred (Shea and Parrish, 1991; Nonaka *et al.*, 1994). The development of sunscreens offers an additional protective mechanism against damage to the skin by UV radiation through its absorption avoiding thus, sunburn, premature aging and skin cancer. Sunscreens are recommended to avoid excessive tanning of the skin, which is nature's sign of skin damage (IARC, 2001). Sunscreens containing both UVA and UVB filters are effective in blocking the cancer-promoting effects of solar UV radiation (Nghiem *et al.*, 2001). In addition, sunscreens have been shown to protect against UVR-induced immune suppression (Ullrich *et al.*, 1999). However, the use of sunscreens alone may not be

sufficient to prevent skin cancer, due to lack of frequent application before each exposure to sunlight and every few hours or after coming into contact with water. Because of these limitations, it could be difficult to completely prevent events such as DNA damage. Further studies are therefore, required in order to gain a better understanding of the genetic and immune suppression pathways involved in UVR-induced carcinogenesis leading to more effective measures in the prevention and treatment of skin cancer.

1.10 PROTECTION FROM INDIRECT DNA DAMAGE

Cells have evolved a variety of defense mechanisms, both enzymatic and non-enzymatic, against the harmful effects of reactive oxygen species resulting thus, in the prevention of oxidative DNA damage. One of these defense mechanisms is antioxidants, which when present at low concentrations compared to that of an oxidizable substrate, result in significantly delay or prevention of oxidation of that substrate (Clarkson, 1995). Antioxidants can act by scavenging biologically important reactive oxygen species including superoxide anion and hydroxyl radical, by binding to them and donating an electron. When a free radical gains the electron from an antioxidant it is no longer attacks the cell resulting in breaking the chain reaction of oxidation (Clarkson, 1995). Thus, antioxidants protect against oxidative stress and prevent damage to cells and may have a role in preventing the formation of cancer (Ames *et al.*, 1995; Halliwell and Gutteridge, 1995).

Enzymatic defence mechanisms involve enzymes, such as glutathione peroxidase, superoxide dismutase and catalase (Clarkson, 1995). Superoxide free radical is simultaneously reduced and oxidized (dismutated) to form hydrogen peroxide and oxygen (as described above in reaction [1]). Superoxide dismutase (SOD) is a very important

enzyme that functions as a cellular anti-oxidant. It is present in cell cytoplasm (copper-zinc enzyme) and in mitochondria (manganese enzyme) in order to maintain a low concentration of superoxide anion. The absence of this enzyme is lethal. The amount of superoxide dismutase is controlled by specific redox-sensitive genes in cells (Halliwell, 1991; Clarkson, 1995).

Similarly, catalase enzyme catalyses the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen, providing thus, a protective role similar to that of glutathione peroxidase as they both remove hydrogen peroxide (Clarkson, 1995).

$$2\,H_2O_2 \xrightarrow{\text{catalase}} O_2 + 2\,H_2O$$

Increased levels of oxidative DNA damage have been shown in haematological disorders like leukaemia through measurement of urinary 8-oxodG levels (Honda *et al.*, 2000) as a result of reduced activities of the antioxidant enzymes catalase, glutathionine peroxidase, and superoxide dismutase. Vitamin E (alpha-tocopherol), Vitamin C (L-ascorbic acid), beta-carotene, and coenzyme Q are all important non-enzymatic antioxidants (Karlsson, 1997). Coenzyme Q is manufactured within the body. Vitamic C and vitamin E as well as beta-carotene can be extracted from the food such as fruits, vegetables, seeds, nuts, meats, and oil. Vitamin E is considered to be essential for the stabilization of biological membranes, particular those containing large amounts of polyunsaturated fatty acids. It is now known that vitamin E acts as an antioxidant, and can inhibit the formation of lipid peroxides (Halliwell and Gutteridge, 1995). It might thus, play a role against aging, particularly of the skin since lipid peroxidation in tissues may be one of the causes of skin

aging (Kaczmarski *et al.*, 1999). It also inhibits apoptosis and cancer formation (Zhang *et al.*, 1997).

Though important in preventing oxidative DNA damage *via* inactivation of free radicals, antioxidants such as vitamin C and E have been reported to have no effects on oxidative DNA damage (Fenech *et al.*, 1997; Prieme *et al.*, 1997). However, several epidemiological studies have shown an opposing association between vitamin C intake, or plasma status, and the risk from different types of cancers (Block, 1991; Enstrom, 1997). Vitamin C has been shown to act as an antioxidant inhibiting the formation of 8-oxodG in Chinese hamster cells following exposure to UV-visible light by HPLC-EC assay (Fischer-Nielsen *et al.*, 1993). Similarly, Noroozi *et al.* (1998) showed inhibition of oxidative DNA damage in human lymphocytes after exposure to hydrogen peroxide by the comet (single cell gel electrophoresis) assay. The antioxidants effects of Vitamin C on oxidative DNA damage have also been determined in human supplementation studies using HPLC-EC assay. Fraga *et al.* (1991) showed significantly decreased levels of 8-oxodG in sperm DNA following Vitamin C supplementation using HPLC-EC.

However, despite this antioxidant effect of Vitamin C, data obtained by Podmore and colleagues (1998) following measurement of two oxidative base lesions, 8-oxoG and 8-oxoA, in peripheral blood lymphocytes by GC-MS assay, showed an antioxidant effect of Vitamin C on oxidative DNA damage in humans, where levels of 8-oxodG were reduced significantly following Vitamin C supplementation. However, in the same study a significant increase in the 8-oxoA levels was reported, suggesting a pro-oxidant effect of Vitamin C in their human study. The data obtained from the above study appeared quite controversial in the field, raising serious issues about the interpretation of the results and

the design of such an experiments (Poulsen *et al.*, 1998b; Levine *et al.*, 1998), mainly due to the generation of artifactual oxidation associated with GC-MS assay and the lack of appropriate control groups throughout the entire study. In addition, the levels of 8-oxoG reported in the above study were approximately 10- to 100-fold higher than those reported by others for human lymphocytes (Collins *et al.*, 1997). The oxidative base lesion 8-oxoA is believed to be at least 10-fold less mutagenic than 8-oxoG (Wood *et al.*, 1992), quite controversial, therefore, with the results obtained by the study described by Podmore *et al.* (1998). Further study by the same group (Cooke *et al.*, 1998) reported an antioxidant effect of Vitamin C on oxidative DNA damage after measurement of 8-oxodG in three biological matrices (urine, serum and DNA) obtained from the same supplemented subjects. In this study, levels of 8-oxodG were shown to be reduced significantly in DNA after measurement with HPCL-EC whereas serum and urine levels of 8-oxodG increased significantly, the latter being measured using a competitive ELISA method. These findings were explained as being the result of an influence of Vitamin C on the enzymes involved in the repair of 8-oxodG from the DNA (Cooke *et al.*, 1998).

The measurement of endogenous oxidative DNA damage has been the focus of intense debate over the last years. Data obtained from the most commonly methods employed for the measurement of oxidative DNA lesions, including HPLC/EC (Helbock *et al.*, 1998), GC/MS (Dizdaroglu and Gajewski, 1990; Jenner *et al.*, 1998), ^{32}P-postlabeling (Povey *et al.*, 1993) showed discrepancy in the results. Cadet *et al.* (1997) reported the levels of 8-oxodG and 8-oxoG to range from one adduct in 10^7 nucleotides to one adduct in 10^3 nucleotides in human tissues. Moreover, the baseline levels of 8-oxodG have been shown to vary by orders of magnitude after measurement of this oxidative base lesion in the same samples by another laboratory (Beckman and Ames, 1999). The differences observed in

the baseline levels of 8-oxodG or 8-oxoG were likely to be the result of technical problems associated with GC-MS assay, in particular, including the generation of artifactual *ex vivo* and *in vitro* oxidation during isolation and extraction of DNA as well as derivatization for GC-MS analysis (Cadet *et al.*, 1997; 1999; Jenner *et al.*, 1998).

To sum up, there is large controversy in the effects of Vitamin C in oxidative damage. Despite the improvements made in GC-MS method to minimize the generation of artifactual oxidation associated with it (Cadet *et al.*, 1997; Jenner *et al.*, 1998), the employment of alternative methods, such as HPLC-EC and the comet assay, for the measurement of oxidative DNA damage might improve analysis of oxidative base lesions, mainly due to the reduced generation of oxidation artifacts (Collins *et al.*, 1997; Helbock *et al.*, 1998). In addition, the use of alternative biomarkers for oxidative stress, rather than just the oxidised base lesions 8-oxodG or 8-oxoG could possibly improve studies analyzing the role of Vitamin C in oxidative damage or oxidative stress in general. The use of single-strand breaks, for example, as a marker of oxidative damage to DNA could be an alternative choice. Though, they are not as specific as 8-xodG and 8-oxoG, their specificity could be improved by the use of lesion-specific glycosylase repair enzymes (Pflaum *et al.*, 1997). Le *et al.* (1998) has also reported the use of thymine glycol as an alternative biomarker of oxidative damage to DNA using a monoclonal antibody specific to thymine glycol residues in DNA.

1.11 REPAIR OF UVR-INDUCED DNA DAMAGE

DNA is constantly subject to damage arising from both endogenous and exogenous DNA damaging agents. Faithful maintainance of the integrity of the genome and accurate transmission of genetic information by DNA is crucial to all living organisms. Cells have

developed efficient DNA repair mechanisms to counteract the mutagenic effects of DNA lesions. These are photoreactivation, excision repair, including base excision repair (BER), nucleotide excision repair (NER), transcription-coupled repair (TCR), mismatch repair (MMR) and recombinational repair.

1.12 REPAIR OF INDIRECT DNA DAMAGE

1.12.1 Base Excision Repair (BER)

BER is a major cellular defence mechanism against endogenous non-bulky DNA lesions (Sancar, 1995). BER is initiated by the action of a glycosylase enzyme, which recognises the damaged or mismatched base and catalyses the *N*-glycosidic bond between the base and the DNA sugar-phosphate backbone resulting in the creation of an apurinic/apyrimidine (AP; abasic) site. This AP site is then processed by an AP endonuclease or an AP lyase that cleaves the phosphodiester bond either 5' or 3' to the AP site respectively (Krokan *et al.*, 1997). Different kinds of damage are recognised by different DNA glycosylases thus, the specificity of the repair pathway is determined by the type of the glycosylase involved in the removal of a DNA lesion (Seeberg *et al.*, 1995). Some glycosylases also cleave the phosphodiester backbone 3' of apurinic/apyrimidinic (AP) sites created by glycosylases *via* their DNA lyase activity (Krokan *et al.*, 1997). In mammalian cells two pathways have been described for the repair of AP sites, a single nucleotide insertion ("short patch") pathway, which is catalysed by DNA polymerase β, and a proliferating cell nuclear antigen (PCNA) dependent ("long patch") pathway, involving a resynthesis patch of 2 up to 13 nucleotides long (Klungland and Lindahl, 1997; Lindahl and Wood, 1999). In the short patch, the resulting 3' hydroxyl residue of the AP site is excised by DNA polymerase β (Polβ) and the nick is sealed by DNA ligase I or III

(Lindahl and Wood, 1999). In the long patch repair pathway, AP site adducts that are resistant to excision by the polβ activity are repaired by polymerases δ and/or ε, which displace the damaged strand resulting in the creation of a 5' flap junction in association with the proliferating cell nuclear antigen (PCNA) and replication factor-C (RF-C) (Klungland and Lindahl, 1997.; Kim *et al.*, 1998). BER is then completed by the activity of the flap endonuclease-1 (FEN-1) enzyme, which cleaves the 5' overhang followed by a sealing of the DNA nick by a DNA ligase (Kim *et al.*, 1998) (Figure 1.6).

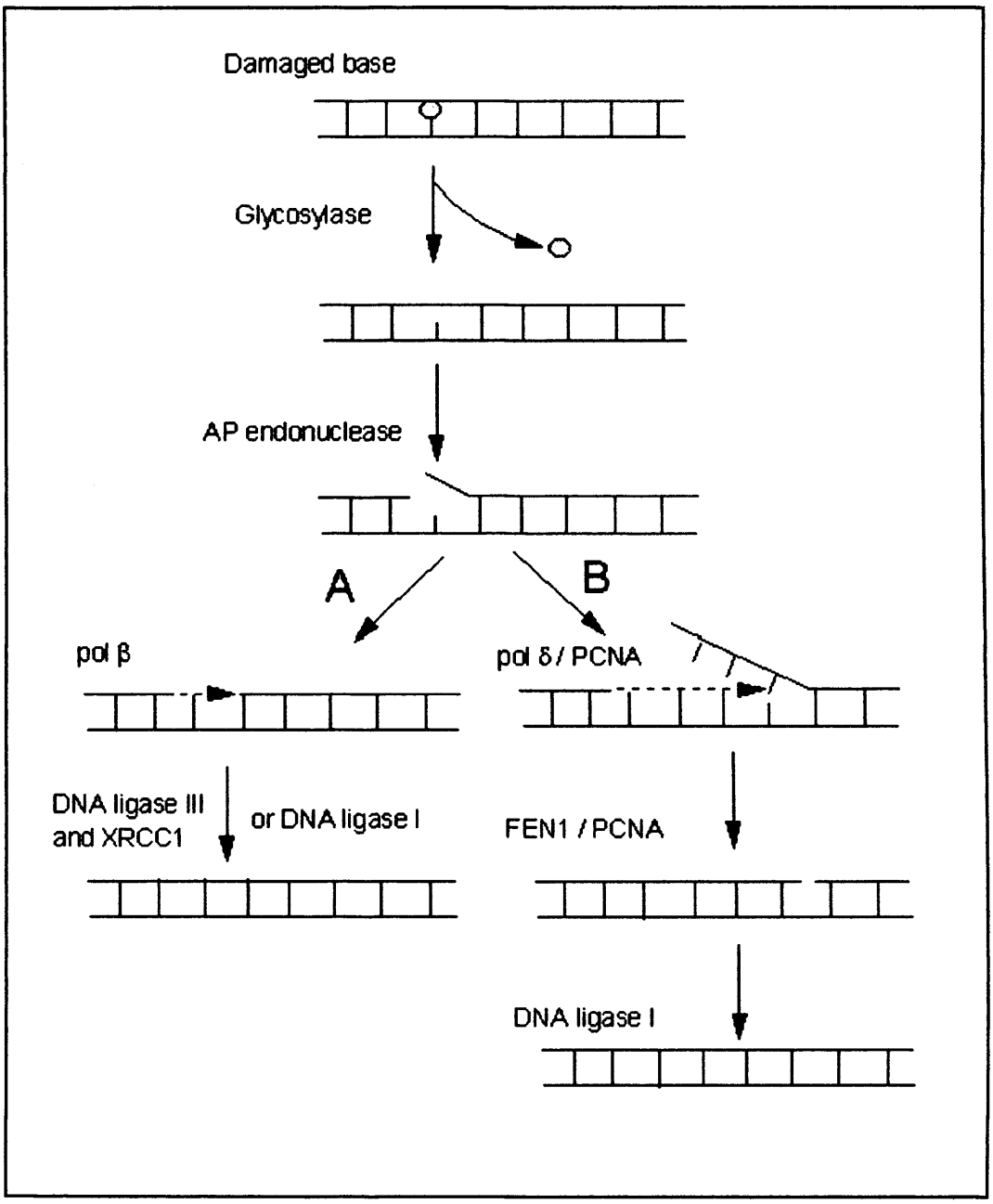


Figure 1.6: Base excision repair pathways. A. Short patch repair pathway results in the excision of a single nucleotide by DNA polymerase β (Polβ) and sealing of the nick by DNA ligase I or III. B. Long patch repair pathway requires proliferating cell nuclear antigen (PCNA) and replication factor-C (RF-C) resulting in a repair patch of 13 nucleotides by excision by polymerases δ and/or ε (adapted from Klungland and Lindahl, 1997).

The repair of oxidative DNA lesion 8-oxoG is performed by two DNA glycosylases, called hOGG1 and hOGG2 (human 8-oxoguanine glycosylases 1 and 2) (Rosenquist *et al.*, 1997; Hazra *et al.*, 1998; Boiteux and Radicella, 2000). The former glycosylase removes misincorporated 8-oxoG opposite a C residue, which was first characterised in *Saccharomyces cerevisiae* by van der Kemp *et al.* (1996), while the removal of 8-oxoG opposite misincorporated adenine or guanine residue is accomplished by hOGG2 (Hazra *et al.*, 1998). Another DNA glycosylase MYH also excises an A residue inserted opposite to an 8-oxoG by glycosylase action (Slupska *et al.*, 1996). This repair system was first defined in *Escherichia coli* by Holmes *et al.* (1992) and following cloning and sequencing of the *Escherischia coli* MutY gene a human homologue (hMutY) was discovered and characterised by Slupska *et al.* (1996).

Oxidative damage to pyrimidines, such as thymine glycol, is removed by a specific DNA glycosylase, called hNth1, which is the human homologue of bacterial endonuclease III (Hilbert *et al.*, 1997; Ikeda *et al.*, 1998; Dizdaroglu *et al.*, 1999). Data from both *in vitro* (Klungland *et al.*, 1999) and *in vivo* (Cooper *et al.*, 1997) studies showed that activation of XPG protein as a loading factor for hNth1 is required in base excision repair of oxidative DNA damage in human cells. DNA glycosylase hNth1 is also involved in the removal of 8-oxoG resulting from 8-oxoG:G mispairs (Matsumoto *et al.*, 2001). In addition, 8-oxoG can be removed by the glycosylase action of the mammalian N-methylpurine DNA glycosylase (MPG), which repairs N-alkylpurine as its main substrate (Bessho *et al.*, 1993). The human thymine mismatched-DNA glycosylase (TDG) is involved in the repair of thymine or uracil opposite G residues resulting in the removal of deaminated 5-methylcytosine residues (Waters and Swann, 1998). In addition, the human uracil DNA *N*-

glycosylase (UNG) was shown to remove 5-hydroxyuracil (5-OHUra) lesion, which is formed as a product of cytosine oxidation (Dizdaroglu *et al.*, 1996).

1.12.2 Nucleotide Excision Repair (NER)

NER has been reported to play a role in the repair of bulky lesions such as UVR-induced CPDs as well as in the removal of non-bulky, oxidative DNA base lesions such as 8-oxoG resulting in lesion-containing oligomers (Huang *et al.*, 1994; Cooper *et al.*, 1997). Reardon *et al.* (1997) showed a faster repair of the oxidative DNA lesion 8-oxoG by NER compared to that of T<>T in his *in vitro* studies, suggesting the physiological importance of NER as a repair pathway of oxidative DNA damage. Products of NER are lesion-containing oligomers about 24 to 32 bases long (Huang *et al.*, 1992). Such excised oligomers are further subjected to 5'→3' exonucleolytic attack that yields oligomers 6 to 7 nucleotides in length or by adequate degradation the 8-oxodG lesion (Galloway *et al.*, 1994). Cooke *et al.* (2001a) showed the presence of 8-oxodG-containing oligomers in urine as a repair product of the NER pathway. However, the role of NER as a major repair pathway of oxidative DNA damage is still under question due to the fact that NER appears to function preferentially in certain types of cells or in situations that BER pathway is compromised (Dianov *et al.*, 1998).

1.12.3 Transcription-Coupled Repair (TCR)

Studies in DNA damage induced by various chemical carcinogens such as UV radiation have revealed that the repair of various DNA adducts including UVR-induced CPDs and (6-4)PPs and oxidative DNA lesions to occur in a preferential manner with a faster removal of lesions being observed in transcriptionally active genes than in inactive regions or the genome as a whole (Bohr *et al.*, 1985; Mellon and Hanawalt, 1989). This rapid

repair was shown to be due to a faster repair of damage in the transcribed strand than in the non-transcribed strand (Mellon *et al.*, 1987; Leadon and Lawrence 1991). TCR acts on lesions that arrest translocation of RNA polymerase II (RNAP II) on the transcribed strand, which provides a signal for rapid induction of TCR to that strand (Mellon *et al.*, 1987). Oxidative base lesions, such as 8-oxoG and Tg, have been reported to block transcription by RNA polymerase *in vitro* (Evans *et al.*, 1993; Hatahet *et al.*, 1994), suggesting the requirement of TCR pathway for their removal from DNA.

Deficiency in TCR of oxidative lesions results in genetic diseases such as the Cockayne syndrome (CS). Cockayne syndrome (CS) is a rare inherited human genetic disorder characterized from postnatal growth failure that results in dwarfism, photosensitivity, skeletal abnormalities, mental retardation and progressive neurological degeneration (Nance and Berry 1992; Friedberg *et al.*, 1995). Patients with CS are associated with mutations in the CSA or CSB genes, which are required for the preferential removal of lesions from the transcribed strand of active genes by the TCR pathway (Leadon and Cooper, 1993; Cooper *et al.*, 1997). Recently, Le Page *et al.* (2000) demonstrated that Tg and 8-oxoG are removed by the TCR pathway that does not involve NER but requires the XPB and XPD components of the basal transcription factor TFIIH as well as XPG and CSB, suggesting TCR as a discrete pathway for the rapid removal of lesions that block transcription and not as a sub-pathway of NER. Furthermore, Gowen *et al.* (1998) showed that mouse embryonic stem cells deficient in BRCA1 are defective in the ability to carry our TCR of oxidative DNA damage, suggesting the participation of BRCA1 gene in the repair of oxidative base lesions, such as Tg, by the TCR pathway.

1.13 REPAIR OF DIRECT UVR-INDUCED DNA DAMAGE

1.13.1 Photoreactivation

This is one of the simplest and perhaps oldest repair systems consisting of a single enzyme called photolyase (Sancar, 1994). The mechanism is named photoreactivation because it occurs in the presence of visible light. Photolyase contains a reduced flavin adenine dinucleotide ($FADH_2$) group, which absorbs photoreactivating light (350 to 500nm), and binds to CPDs. Upon binding to CPDs, the dimerised dimers are reversed to their monomeric form by an electron transfer from the $FADH_2$ at the active site of the photolyase to the dimers resulting in the breakage of the pyrimidine bond (Sancar, 1994; Thoma, 1999).

Photoreactivation has been reported to occur in many bacteria, plants, fungi, fish and insects but seems to be absent or non-functional in humans (Sancar, 1996; Todo, 1999). While there is evidence of photoreactivating activity in human white blood cells (Sutherland and Bennett, 1995) and fibroblasts (Zölzer *et al.*, 1993), its presence in humans is still under question (de Gruijl and Roza, 1991; Ley, 1993). However, the discovery of a human homologue of a *Drosophila* photolyase gene with unknown function could provide a possibility of the existence of photoreactivation in humans (Kanai *et al.*, 1997; Todo *et al.*, 1997). A distinct photolyase specific for the reversal of (6-4)PPs was detected in *Drosophila* cell-free extracts, called 6-4 photolyase (Todo *et al.*, 1993) and this was further supported by Kim *et al.*, 1994. In addition, evidence from both *in vivo* (Yamamoto *et al.*, 1983; Sancar and Smith, 1989) and *in vitro* (Sancar *et al.*, 1984) studies showed binding of photolyase to CPDs, even in the absence of light, stimulating the removal of CPDs by the NER pathway.

1.13.2 NER for UVR-induced DNA damage

NER is a versatile and highly conserved repair pathway capable of removing helix-distorting DNA lesions, such as UVR-induced CPDs and (6-4)PPs and other bulky adducts such as those derived from cisplatin, aflatoxin and benzopyrene (Wood, 1996; Sancar, 1996). The basic mechanism of nucleotide excision repair includes (i) damage recognition, (ii) assembly of repair factors at the site of damage, (iii) dual incisions that result in excision of damage-containing oligomers, (iv) resynthesis to fill in the gap, and (v) ligation. In the first step, a protein complex XPC-HHR23B recognises the DNA damage site and binds to it initiating NER (Sugasawa *et al.*, 1998). In the second step, DNA unwinds and the lesion is opened by the action of XPA protein, replication protein A (RPA), and the bi-directional XPB/XPD helicase subunits of the transcription factor IIH (TFIIH) complex (Wood, 1996; Evans *et al.*, 1997b). Specific nucleases, XPG and ERCC1-XPF, are then cleaved the damaged strand on the 3' side and on the 5' side of the lesion, respectively, releasing an oligonucleotide 24-32 residues long (Mu *et al.*, 1996). Finally, repair synthesis is accomplished by mammalian DNA replication factors, such as proliferating cell nuclear antigen (PCNA), replication factor C (RF-C), RPA, and DNA polymerase δ and ε holoenzyme. The reaction is completed by ligation of a new synthesised DNA by a DNA ligase, probably LIG1 (Thoma, 1999; Nocentini, 1999; Matsumura and Ananthaswamy, 2002) (Figure 1.7).

Defects in repair genes result in three distinct sun-sensitive, cancer-prone genetic disorders including Xeroderma pigmentosum (XP), Cockayne syndrome and trichothiodystrophy (TTD) in humans (Friedberg *et al.*, 1995; Cleaver and Kraemer, 1995). Patients with xeroderma pigmentosum (XP) have an approximate 1000-fold increase of developing skin cancer (Cleaver and Kraemer, 1995) resulting from defects in

one the seven human XP genes (XPA through XPG) that inactivate the NER process (Cleaver and Kraemer, 1995; Thompson, 1998). The variant form of XP (XPV) is characterized by a cellular defect in the ability of cells to synthesize intact daughter DNA strands on damaged templates, arising from mutations in the gene encoding polymerase η, which is required for translesion synthesis (TLS) past damaged sites (Broughton *et al.*, 1995, Svoboda *et al.*, 1998).

The second sun-sensitive disorder, Cockayne syndrome (CS) arises from mutations in the CSA or CSB genes required for preferential removal of UV-induced lesions in template strands of genes transcribed by RNA polymerase II in the TCR process (Hanawalt, 1994; Thompson, 1998). Finally, patients with trichothiodystrophy (TTD) have genes that are defective in XP complementation group D (Botta *et al.*, 1998; De Boer and Hoeijmakers, 2000).

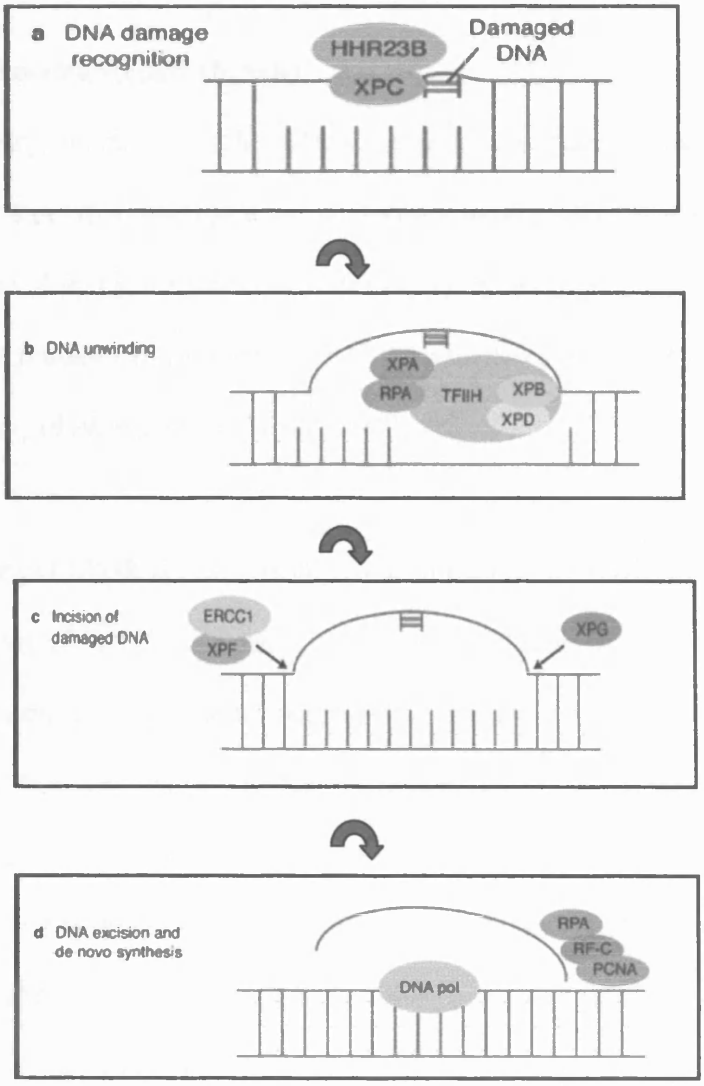


Figure 1.7: Mammalian NER pathway. (a) In the first step, DNA damage recognition is performed by XPC/HHR23B protein complex, which binds to the damaged DNA site initiating NER. (b) DNA unwinds and the lesion is opened by the concerted action of XPA protein, replication protein A (RPA), and the bi-directional XPB/XPD helicase subunits of the transcription factor IIH (TFIIH) complex. (c) Specific nucleases, XPG and ERCC1-XPF, are then cleaved the damaged strand on the 3' side and on the 5' side of the lesion, respectively. (d) Repair synthesis is performed by mammalian DNA replication factors such as proliferating cell nuclear antigen (PCNA), RPA), replication factor C (RF-C), RPA and DNA polymerase δ and ε holoenzyme. The reaction is completed by ligation of the newly synthesised DNA by a DNA ligase, probably LIG1 (adapted and modified from Matsumura and Ananthaswamy, 2002).

1.13.3 Mismatch Repair (MMR)

The primary function of the MMR repair is to remove base-base mismatches and insertion/deletion loops that arise as a consequence of base misincorporation and DNA polymerase slippage, which occurs during DNA synthesis, resulting in the generation of permanent mutations in the genome of the affected cell, if left unrepaired (Kolodner and Marsischky, 1999; Buermeyer *et al.*, 1999).

The process of MMR is best characterized in *Escherichia coli* (*E. coli*) (Marti *et al.*, 2002). Homologues of the *E. coli* mutS gene and mutL gene, termed MSH and MLH or PMS genes respectively, have been identified in humans (Fishel and Wilson, 1997; Peltomäki and de la Chapelle, 1997). The human homologues of MutS and MutL genes function as heterodimers comprised of different MutL (MLH1, MLH3, and PMS1 or PMS2) or MutS (MSH2, and MSH3 or MSH6) homologues (Kolodner and Marsischky, 1999; Fishel and Wilson, 1997). The different pairing combinations appear to have an effect on the specific activities of the complexes. The MSH2/MSH3 heterodimer has a low affinity for single base mismatches, such G:T or O^6-methylguanine, but a high affinity for single stranded loops arising from strand slippage during replication. This affinity increases with the size of the single stranded loop (Palombo *et al.*, 1996). In contrast, the MSH2/MSH6 heterodimer, which appears to be the predominant form, exhibits a high affinity for single base mispairs, and single stranded loops arising from the insertion/deletion of only one nucleotide (Fishel and Wilson, 1997). For mismatch recognition, the human MSH2 protein forms a complex with MSH6 or MSH3, termed MutSa or MutSβ heterodimer respectively (Palombo *et al.*, 1996; Fishel and Wilson, 1997). Following binding of MSH2 and MSH6 or MSH3, a heterodimer of MLH1 and PMS2 or MLH3 or PMS1 bind to each other and the recognition complex (MSH2 and MSH6 or MSH3) synchronizing the recognition

complex with other proteins involved in the MMR process most probably the proliferating cell nuclear antigen (PCNA), exonucleases, DNA polymerases, such as β or ε, replication factors including single-stranded DNA-binding protein RPA, and probably helicases. (Peltomäki, 2001).

In addition to its primary role in correcting base mispairs, MMR proteins are involved in the response of cells to a variety of DNA lesions induced by exogenous and endogenous sources. Indeed, repair proteins of the MMR process have been shown to recognise oxidative UVA-induced DNA base lesions, such as 8-oxoG and Tg (Mazurek *et al.*, 2002). Data from several studies have also revealed an increase in levels of mutations caused by oxidized bases in MMR-defective cells (Jackson *et al.*, 1998; Ni *et al.*, 1999; Mazurek *et al.*, 2002). Furthermore, MMR proteins have been shown to facilitate cell cycle arrest especially in the G_2-M phase of the cell cycle (Hawn *et al.*, 1995; Davis *et al.*, 1998) and apoptosis (Gong *et al.*, 1999; Hickman and Samson, 1999; Hardman *et al.*, 2001). Overexpression of human MSH2 or MLH1 MMR proteins could also lead to the induction of apoptosis in mammalian cells (Zhang *et al.*, 1999).

Though important in removing DNA base lesions formed by oxidative damage to DNA, the role of MMR proteins in the repair of UVR-induced photoproducts is still under question. However, Wang *et al.* (1999) showed specific binding of the human MutSa heterodimer (MSH2 and MSH6) for CPDs and (6-4)PPs opposite mismatched bases. Recent data by Young *et al.* (2003) showed MSH6 mismatch repair protein to be essential in initiating effective apoptosis in primary mouse fibroblast cells following exposure to UVB radiation. Similarly, Peters *et al.* (2003) showed induction of apoptosis in cells after

exposure to UVB-induced DNA damage due to mammalian MMR proteins. Thus, although the contribution of MMR proteins in the repair of UV-induced DNA photoproducts is not effective, MMR proteins seem to be important in reducing mutagenesis via the induction of apoptosis and cell cycle arrest.

Defects in the MMR pathway lead to hereditary nonpolyposis colon cancer (HNPCC) arising from mutations in one of the MMR genes, particularly MSH2, MSH6, MLH1, PMS1 and PMS2 (Liu *et al.*, 1996). Further analysis has shown that the majority of HNPCC cases have mutations in MLH1 or MSH2, and only a few mutations are seen in MSH3, MSH6, PMS1, and PMS2 (Nicolaides *et al.*, 1994; Peltomäki and Vasen, 1997). Cells that are deficient in the MMR pathway accumulate somatic mutations in proto-oncogenes and tumour suppressor genes (Vogelstein and Kinzler, 1993), which leads to defective proofing of DNA following replication and as a consequence to an accumulation of multiple mutations, a prerequisite for tumorigenesis. Thus, MMR genes are important to the maintenance of genomic stability and the prevention of cancer.

Other types of sporadic cancer including ovarian, endometrial, cell lung, pancreatic, gastric, cervical, and breast carcinomas are associated with defective MMR genes (Wooster *et al.*, 1994). An increase in the resistance to DNA damage from chemotherapeutic agents like procarbazine, temozolomide, busulfan, cisplatin, and carboplatin was shown in cells with faulty MMR (Fink *et al.*, 1998).

1.14 OTHER REPAIR PATHWAYS

1.14.1 Recombinational Repair

Recombination is a very important process involved in the repair of single-strand breaks (SSBs) and double-stand breaks (DSBs) in damaged DNA. Single-strand breaks do not represent a great threat to the cell as they are removed using the same enzymes that are used in BER pathway (Friedberg *et al.*, 1995). However, double-strand breaks are the primary cytotoxic lesion induced by ionizing radiation and endogenous oxidative damage, leading to chromosomal deletion, rearrangement, loss or cytotoxicity if not repaired immediately (Friedberg *et al.*, 1995; Shinohara and Ogawa, 1995). Two major mechanisms exist for the repair of DSBs, non-homologous end joining (NHEJ), and homologous recombination (or recombinational repair (RR).

NHEJ accounts for the repair of most of DSB in the G1 phase and involves the direct rejoining of the separated DNA ends (Hendrickson, 1997). This requires little or no sequence homology between the broken DNA ends (Kannar *et al.*, 1998; Jeggo, 1998). A protein called Ku, which is a heterodimer of Ku70 and Ku80 subunits, is essential in this process as it recognizes the DSB and binds to it (Boulton and Jackson, 1998). Upon binding a DSB, the Ku heterodimer attracts the catalytic domain of the DNA-dependent protein kinase (DNA-PKcs) (Yaneva *et al.*, 1997; Hammarsten and Chu, 1998). This complex is then joined by another complex RAD50, MRE11 and p95, which may play a role in processing the DNA ends. Finally the DNA ends are rejoined by a complex of XRCC4 and DNA ligase IV (Hendrickson, 1997; Barnes, 2001). However, the process is inherently error-prone as nucleotides at the break could be added or lost, and incorrect ends might be joined.

In contrast to NHEJ, RR uses homologous DNA as a template in rejoining DSBs, allowing damaged DNA ends to be both repaired and appropriately re-joined (Kannar *et al.*, 1998). However, humans contain high numbers of repetitive DNA sequences, which may be incorrectly used by RR as homologous sequences in repairing DSBs. Consequently, RR is only active at times in the cell cycle after the cell has duplicated its DNA, but before it has divided (late S through early M phases of the cell cycle) (Hendrickson,1997). This provides RR with nearby identical DNA sequences to use in repairing DSBs. Thus, NHEJ is important in the G1 phase of the cell cycle, while homologous recombination accounts for most DSB repair in the late S/G2 phases (Hendrickson, 1997). In homologous recombination, the DNA ends resulting from a DSB are believed to be processed by nucleases and/or helicases to generate a single stranded 3' overhang (Kannar *et al.*, 1998; Jeggo, 1998).

1.15 HETEROGENEITY OF DNA DAMAGE AND REPAIR

Most of the work in DNA damage and repair over the last decades has been done by measuring the events in the total genome. However, studies on carcinogens, such as UV radiation, have shown that induction of DNA damage and repair does not occur equally throughout the entire genome (Bohr *et al.*, 1985), but varies according to factors such as DNA sequence, chromatin structure, characteristics of the proteins involved in the repair of DNA damage and transcriptional activity of actively expressed genes (Mellon *et al.*, 1987; Bohr *et al.*, 1987; Pfeifer, 1997). Ultraviolet-light (UV)-induced pyrimidine dimers, for example, are repaired more efficiently in actively transcribed genes as compared to inactive genomic regions or the overall genome (Bohr *et al.*, 1985; 1987; Mellon *et al.*, 1986; Hanawalt, 1989). This gene-specific repair is probably due to the rapid repair of the transcribed strand of actively expressed genes (Hanawalt, 1989). The preferential repair of

transcribed genes has been shown to correlate with the sensitivity of cells to various DNA damaging agents, such as UV radiation (Bohr *et al.*, 1985; 1987).

Accumulation of DNA damage in functionally important regions such as replication origins, promoters or essential genes can influence normal cellular functions. Since the genes only constitute a small fraction of the overall genome, any changes in gene-specific repair would not be detected using traditional assays at the level of the overall genome (Bohr *et al.*, 1985). Thus, localisation of specific damage and quantification of repair within specific genes may provide an improved insight into the aetiology and treatment of malignancies, compared to genome level measurements.

1.16 METHODS FOR THE DETECTION OF DNA DAMAGE

The current emphasis in the area of DNA damage and repair is on the structure of the DNA adducts, their detection and quantification in cellular DNA, their fate in specific mammalian cell types, and analysis of damage in specific genomic segments, particularly the loci that are relevant targets in mammalian oncogenic transformation (Singer and Grunberger, 1983; Bohr *et al.*, 1986).

Several methods have been developed for the detection and quantification of DNA lesions formed by UV radiation and oxidative DNA damage. The ^{32}P-postlabeling method is an important assay that is widely employed for the measurement of cyclobutane T<>T dimers (Bycov *et al.*, 1995) and several other types of DNA lesions, including thymidine glycol (Hegi *et al.*, 1989) and 8-oxodG (Povey *et al.*, 1993). The radiolabelled compounds can be separated either by multi-dimensional thin layer chromatography (TLC) or high-performance liquid chromatography with radioactivity detection (^{32}P-HPLC) (Mőller *et al.*,

1993). The snake venom phosphodiesterase-based ^{32}P-postlabeling procedure (SVPD-postlabeling) is an alternative post-labeling protocol that has been successfully applied to the detection of oxidative base lesions, including thymine glycols and phosphoglycolates (deoxyribose fragments present at the 3' termini of oxidative strand breaks), induced by the action of ionising radiation and oxidative stress (Weinfeld and Soderlind, 1991; Bertocini and Meneghini, 1995), and also abasic sites (Weinfeld *et al.*, 1990). SVPD-post-labeling has also been exploited in the analysis of UV-induced photoproducts (Weinfeld *et al.*, 1989; Bycov *et al.*, 1998). Jones and colleagues (1999) have demonstrated that the SVPD-post-labeling method negates the problem of adventitious oxidative effects during ^{32}P-labeling and its application in the biomonitoring of endogenous oxidative stress in human lymphocyte DNA (Routledge *et al.*, 1998). High performance liquid chromatography (HPLC) is another important technique that has been applied to the quantification of T<>T within isolated and radiolabelled cellular DNA, following formic acid treatment (Cadet *et al.*, 1983) as well as in the identification and quantification of thymine glycol in γ-irradiated DNA (Frenkel *et al.*, 1981). Using the same hydrolysis procedure, the formation of *cis-syn* T<>T has been monitored in UV-irradiated DNA by gas chromatography-mass spectrometry (GC-MS) (Podmore *et al.*, 1996). GC-MS method has also been applied in the quantification of 8-oxoguanine as the free base (Dizdaroglu, 1993). UV-induced thymine dimeric lesions have also been detected within isolated and cellular DNA using HPLC with electrospray ionisation tandem mass spectrometry (HPLC-MS/MS) (Douki *et al.*, 2000). Floyd *et al.* (1986) was demonstrated the detection of 8-oxo-2'-deoxyguanosine using HPLC with electrochemical detection (HPLC-EC). Though important, all these techniques have limitations, including extensive sample preparation for DNA isolation and derivatisation of products leading to the generation of artefacts, poor labelling efficiency

and radiolytic autooxidation as well as poor chromatographic resolution of the products and perhaps, most significantly the inability to localise DNA damage in specific genes.

Lesion-specific enzymes, such as T4 endonuclease V (Seawell *et al.*, 1980), and UV-endonuclease from *Micrococcus luteus* (Sutherland and Shih, 1983) have been used in the quantification of CPD by electrophoresis in alkaline agarose gels. However, this assay is associated with limitations including the use of suitable restriction enzyme recognition sites within the region of interest, the requirement of large amounts of DNA and the generation of single strand break of the enzyme may be incomplete. Polymerase chain reaction (PCR) can be used as an alternative method for measuring gene-specific DNA damage and repair (Govan *et al.*, 1990; Jennerwein and Eastman, 1991; Kalinowski *et al.*, 1992) and has the advantage of not requiring specific restriction endonucleases to recognise lesions. Although very sensitive, large amounts of lesions in the target DNA segment are required in order to obtain a quantifiable decrease in amplification. Ligation-mediated polymerase chain reaction (LMPCR) is another sensitive method for detecting and mapping the frequency of rare DNA single-stranded breaks along a complex genome (Műller and Wold, 1989; Pfeifer *et al.*, 1991) and has been applied to map reactive oxygen species-induced DNA damage (Rodriquez *et al.*, 1995; 1997), UV-induced DNA damage (Tornaletti *et al.*, 1993; Gao *et al.*, 1994; Tornaletti and Pfeifer, 1994), and mutagen-induced DNA damage, particularly (6-4) photoproducts (Denissenko *et al.*, 1996a) at single nucleotide resolution. DNA strand breaks can be mapped as long as strand break induction results in a 5'phosphoryl end by chemical or enzymatic means. Quantification of DNA damage can be performed by separating PCR amplified DNA fragments on a sequencing polyacrylamide gel, followed by transferring them to a nylon membrane by electroblotting and analysis of the resulting autoradiogram (Pfeifer *et al.*, 1991; Rodriquez

et al., 1995). However, this technique is limited by the requirement for high doses of mutagens, which are often cytotoxic to mammalian cells, and appears to be most suited for mapping lesions, and not for quantification. The alkaline comet assay (single cell gel electrophoresis) is another sensitive and rapid method that can be used to measure single-strand breaks and alkali-labile sites in individual cells (Fairbairn *et al.*, 1995; Collins *et al.*, 1997). Measurement of DNA strand breaks can be achieved by electrophoresis of a slide containing a suspension of single cells in agarose and staining of the slides with a fluorescent DNA binding stain for computer-assisted image analysis to integrate fluorescence intensity (Collins *et al.*, 1997). The comet assay can also be used to assess the efficacy of enzymatic DNA repair processes, which involve the incision, and subsequent rejoining of damaged strands (Singh *et al.*, 1988). It can, therefore, provide valuable information regarding the molecular mechanisms that counteract radiation insult (Alapetite *et al.*, 1996). In addition, the comet assay has been applied for the measurement of DNA damage in human cells following exposure to UV radiation (Rűnger *et al.*, 2000). UVR-induced comets can be detected as strand breaks resulted from the incision step of excision repair at DNA photoproducts (Rűnger *et al.*, 2000).

The immunological detection of DNA lesions provides an alternative to the chromatographic and enzymatic methods used to measure these lesions. Polyclonal and monoclonal antibodies raised against numerous forms of base damage, such as specific UV-induced lesions, for instance cyclobutane thymine dimers and (6-4) photoproducts (Műller and Rajewsky, 1980; Blount *et al.*, 1990; Mori *et al.*, 1991; Herbert *et al.*, 1994), alkylated guanine residues (Prevost *et al.*, 1990), 8-oxodeoxyguanosine (Kasai and Nishimura, 1986; Park *et al.*, 1992) have been applied to the detection and quantification of DNA adducts. Immunological assays represent a convenient approach, which however,

could suffer from lack of calibration since individual measurement of each class of photoproducts is not possible preventing thus, accurate quantitative measurements.

The employment of antibodies for the quantification of DNA damage within discrete target sequences has been established using sequence-specific ALISS (adduct levels in specific sequences) assay (Hochleitner *et al.*, 1991; Thomale *et al.*, 1994) and immuno-coupled PCR (ICPCR) (Denissenko *et al.*, 1994; 1996b). ICPCR assay is an assay that utilises the sensitivity of antibody-antigen interaction to specifically retain damage-containing DNA fragments prior to the amplification of known gene sequences in these fragments by quantitative PCR (qPCR) (Wani and Arezina, 1991; Kalinowski *et al.*, 1992; Denissenko *et al.*, 1994; 1996b). Although in other PCR-based methods quantification was based on the measurement of decrease in the amount of PCR signal from the modified template (Govan *et al.*, 1990; Jennerwein and Eastman, 1991; Kalinowski *et al.*, 1992), in ICPCR assay quantification of damage is possible on the basis that the amount of PCR product is proportional to the initial amount of damage. Unlike Southern hybridisation assay (Bohr *et al.*, 1985), this assay does not require introduction of single strand breaks and is able to measure repair in very small amounts (nanograms) of genomic DNA. Clearly this assay is of importance, perhaps providing a better measurement of intermediate markers of disease compared to total damage measurements. It is likely to be applicable to any antibody, given an appropriate degree of specificity and hence has a broad range of fields in which it may be applied.

UV radiation is a potent DNA-damaging agent and a known inducer of skin cancer in humans. Heterogeneity of DNA damage induction and repair along the whole genome necessitates the employment of assays to quantify DNA damage and repair in specific gene

sequences, as there are likely to provide an insight into the aetiology and treatment of malignancies, compared to genome level measurements. Continued efforts in the research of skin cancer may help to increase overall awareness of the harmful effects of UV exposure and result in better methods of skin cancer prevention and treatment.

1.17 AIMS OF THE THESIS

The major aim of this thesis is the assessment and quantification of DNA damage and repair induced in naked DNA and cultured human cells following exposure to UVC and UVB radiation. From this, a comparison between levels of DNA damage and repair in gene-specific sequences and in the global genome is performed in an attempt to better understand the involvement of UV radiation in the distribution of damage and repair in genomic DNA.

Essential to this objective was the development of methods for the analysis of DNA damage and repair following UV irradiation, suitable for use in naked DNA and cultured human keratinocyte cells. In order to accomplish this, the following experiments were established:

1. To fully characterise the sequence specificity of a polyclonal antiserum, which was raised against DNA containing thymine-thymine cyclobutane dimers, and to apply the antibody in the detection of UVR-induced DNA damage and repair in specific genes and the global genome.

2. To employ the polyclonal antiserum in the measurement of the repair of thymine-thymine cyclobutane dimers and 8-oxo-2'deoxyguanosine in clinical samples following narrow-band UVB-phototherapy and to subsequently draw conclusions and/or evidence relating to the safety of therapy and mechanism of action.

3. To apply an immunocapture assay, immuno-coupled PCR (ICPCR), in the measurement of cyclobutane thymine-thymine dimers following UVC irradiation of naked DNA in order

to correlate the induction of DNA damage between gene-specific sequences and the global genome, and to subsequently establish the sensitivity and specificity of the ICPCR assay.

4. To quantify the induction and repair of cyclobutane thymine-thymine dimers in human cultured keratinocytes following irradiation to UVB and to compare global-damage assessments to gene-level events.

CHAPTER 2: MATERIALS AND METHODS

CHAPTER 2

2.1 MATERIALS

2.1.1 General chemicals

All chemicals were purchased as the highest purity. The following reagents were obtained from Sigma (Sigma-Aldrich Chemical Company, Poole, U.K.): phosphate-buffered saline (PBS, 0.01M. pH 7.4; was prepared from the tablet form); Tween 20; sulphuric acid (2M; to stop the peroxidase reaction in ELISA assay); phosphate-citrate buffer with sodium perborate capsules (capsule's contents: 0.05 M phosphate-citrate buffer, pH 5.0, containing 0.03% sodium perborate); orthophenylenediamine dihydrochloride (OPD); poly-L-lysine hydrobromide; sodium acetate; ethidium bromide (10mg/ml); protein A from S. *aureus* (Cowan strain) cell walls (100μg/ml); sodium chloride; trizma hydrochloride; trizma base; boric acid; ethylenediamine tetra-acetic acid, dehydrate; dimethylsulfoxide; potassium chloride; magnesium chloride; ammonium sulphate; sodium bicarbonate buffer; glycine; thimerosal; antifoam A; gelatine; polythymidylic acid; 4'-6-diamidino-2-phenylindole (DAPI); MTT [3-(4,5-dimethylthiazol-2-γ2)-2,5-diphenyl tetrazolium bromide].

Fisher Scientific Ltd. (Loughborough, U.K.) was the source of ethanol; methanol and isopropanol, all of HPLC grade.

N,O-bis (trismethylsilyl) trifloroacetamide (BSTFA) and silylation grade acetonitrile were from Pierce and Warriner (Chester, Cheshire, U.K.). Thymine-α- α- α, 6-$^{2}H_4$ was obtained from MSD Isotopes (Montreal, Canada). Formic acid (99%) was from Aldrich Chemical Co. (Gillingham, Dorset, U.K.).

2.1.2 General materials

Calf thymus DNA was obtained from Calbiochem (Nottingham, UK) and only samples with an absorbance ratio at A_{260}/A_{280} greater than or equal to 1.8 were used. Human genomic DNA (type XIII), from human placenta was purchased from Sigma (Sigma-Aldrich Chemical Company, Poole, U.K.). Oligonucleotide primers for PCR reaction, synthetic oligonucleotides containing solely two, four and ten thymines and thymine-containing oligonucleotides of specified sequence were synthesised on an automated DNA synthesiser (ABI 394) and were purchased from the Protein and Nucleic Acid Laboratory, Department of Biochemistry, University of Leicester, U.K. Wizard® Genomic DNA Purification Kit (Cat. # A1120) for the isolation of genomic DNA from tissue culture cells was purchased from Promega (Southampton, U.K.). 8-hydroxy-2'-deoxyguanosine (8-oxodG) ELISA kit was from JaICA (Japan Institute for the Control of Aging; Fukuroi City, Japan). Deoxynucleotides (dATP, dTTP, dCTP, dGTP, 200μM each) were from Sigma. Brilliant® SYBR® Green Quantitative PCR core reagent kit was from Stratagene (La Jolla, CA). Gene Amp® polypropylene tubes (0.5ml) were from Perkin-Elmer (Beaconsfield, U.K.). All plastics for polymerase chain reaction (0.5ml centrifuge tubes, RNase/DNase free sterile tips) were from Helena Biosciences (Sunderland, Tyne and Wear, U.K.). Life Technologies Ltd. (Paisley, Scotland) was the source of 96-well Nunc, Immuno Maxisorp ELISA plates and tissue culture plastics; Nuclease-free PCR grade water; ultra-pure agarose; 123 bp DNA Ladder (1.0μg/μl) and gel loading buffer [0.05% (w/v) bromophenol blue; 40% (w/v) sucrose; 0.1M EDTA, pH 8.0; 0.5% (w/v) sodium lauryl sulfate (SDS)]. Dried skimmed milk was provided by Tesco Stores Ltd (Cheshunt, UK). Methylated bovine serum albumin (BSA) and Freund's Complete Adjuvant were purchased from Sigma.

2.1.3 Equipment

Ultraviolet C (UVC) radiation was emitted from a UVC lamp (254 ± 1nm; Anderman Company, East Molesey, UK; Model-UVGL-58), with a maximum output (λ_{max}) at 254 nm of 10.5μW/cm^2 (at 1m). UVB (λ_{max} 302nm) irradiations were performed by Model-UVM-57 Chromatovue lamp, which was purchased from Knight Optical Technologies (Leatherhead, Surrey, UK). Measurements of irradiance were performed using a UVC (MP-125) and UVB (MP-131) sensor, which were calibrated and provided by Knight Optical Technologies, in conjunction with the optical radiometer (MP-100). Although the employment of a UVC source for irradiation of DNA is not of biological importance, as wavelengths shorter than approximately 290nm do not reach the earth's surface due to absorption by stratospheric ozone (Gasparro and Brown, 2000), the use of a UVC emitting source was suitable in testing the antiserum's antigenicity by ELISA assay and in the development of an immunoassay due to its effectiveness at inducing thymine dimers. In addition, the UVB source, as reported by the manufacturer, showed contamination from UVA and UVC regions (<2% UVC; 95% UVB, 5% UVA) of the spectrum, but this was considered to be minimal based on the reproducibility of the results, making it suitable for use in the induction of cyclobutane thymine dimers in human genomic DNA and in cultured cells. Narrow band ultraviolet light was emitted from 16 UVB lamps (Philips TL-01; 1,80m length, 100W power) with a UVB emission peak at 311nm and was purchased from Verre&Quartz Dixwell, France. Spectrophotometric measurements were made on Shimadzu UV-12DI spectrophotometer. Polymerase chain reaction (PCR) was performed using PCH-3 Dri-Block® Cycler (Techne Corporation, U.K.). Real-time PCR was performed using MX4000® Multiplex Quantitative PCR System from Stratagene (La Jolla, CA). Immunocytochemistry was performed using Zeiss Axioskop fluorescence microscope (Zeiss, U.K.). Haemocytometer was from Weber Scientific International Ltd. (Middlesex,

U.K.). Microscope (Nikon TMS) was obtained from Glenfield Hospital, Leicester, U.K. The gas chromatography-mass spectrometry was performed using using a Perkin-Elmer Autosystem gas chromatograph coupled with a Perkin-Elmer Q-mass 910 quadrupole mass spectrometer. Bio-Rad Laboratories Ltd. (Hertfordshire, U.K.) was the source for the gel tank, gel trays and mini combs used in agarose gel electrophoresis.

2.1.4 Enzymes

HotStarTaq DNA polymerase (5.0 Units/ml) was from Qiagen Ltd. (West Sussex, UK). *Bacillus amyloliquefaciens* H (*Bam*HI) was from Sigma.

2.1.5 Immunochemicals

The polyclonal antiserum 479 (rabbit IgG molecule) was raised against UVC-irradiated polythymidylic acid [UVC-poly(dT)]. Peroxidase-labelled goat anti-rabbit IgG secondary antibody was obtained from Dako (High Wycombe, UK). Fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit secondary antibody was purchased from Sigma-Aldrich Chemical Company, Poole, U.K. Purification of the immunoglobulin G (IgG) fraction of the antiserum was carried out using an affinity chromatography kit (MAbTrap® G II kit) purchased from Amersham-Pharmacia Biotech., St. Albans, Hertfordshire, UK..

2.1.6 Clinical samples

The antigenicity of polyclonal antiserum IgG479 to recognize UVB-induced DNA photoproducts was investigated using urine samples from psoriatic patients. Thirteen patients (7 female and 6 male), age range 19 to 65 years, were recruited from the Department of Dermatology (Leicester Royal Infirmary, Leicester, U.K.) following written, informed consent. Only subjects with skin types I (n=1), II (n=9) and III (n=3)

(Fitzpatrick phototype, 1988) were included. All patients were diagnosed with chronic plaque or guttate psoriasis [Pre-treatment Psoriasis Assessment Severity Index (PASI) score ranged from 4-10.2 (mean 6.8)] and all were on topical therapy comprising emollients (moisturisers). They all had been prescribed TL-01 UVB phototherapy for either chronic plaque or guttate psoriasis. Exclusion criteria were treatment with phototherapy within 3 months of the study, and any history of skin malignancy. All patients stopped any topical therapies two weeks prior to starting TL-01, although emollients were permitted throughout the duration of the study. Ethical approval was granted by Leicestershire Area Health Authority Ethics Committee.

2.1.7 Keratinocyte culture

Spontaneously immortalised human keratinocytes (HaCaT cells, passage #15) were a kind gift from Professor N. E. Fusenig (Deutsch Krebsforschungzentrum, Heidelberg, Germany) (Fusenig *et al.*, 1987; Boukamp *et al.*, 1988). Gibco™-Invitrogen Ltd. (Paisley, Scotland) was the source of Dulbecco's Modified Eagle Medium (D-MEM) (1X) with L-Glutamine, 1000mg/l D-Glucose and Sodium Pyruvate. D-MEM for the culture of HaCat cells was consisted of 10% foetal bovine serum, penicillin-streptomycin (5,000 units of penicillin and 5,000µg of streptomycin/ml utilizing penicillin G (sodium salt) and streptomycin sulfate in 0.85% saline) and 1% Glutamax (GlutaMAX™-I Supplement, 200mM with L-Alanyl-L-Glutamine). Cell culture freezing medium, dimethylsulfoxide (DMSO), trypsin (10X) (2.5g of Trypsin (1:250)/L) and Hank's Balanced Salt Solution (HBSS) (1X) without calcium and magnesium were all purchased from Gibco™-Invitrogen Ltd. Tissue culture plastics were obtained from Helena Biosciences. Lab-Tek™ II eight-well chambered cover glasses were obtained from Nalge Nunc International (Hereford, U.K.). Vector Laboratories, Inc. (Burlingame, CA) was the source of

vectashield mounting medium (H-1000, 10ml). Microscope cover glasses (24 x 60mm) for immunocytochemical staining were from Erie Scientific (Portsmouth, N.H., U.S.A.). Normal goat serum was obtained from DakoCytomation Ltd. (Cambridgeshire, U.K.). Trypan Blue stain (0.4% w/v in 0.8% w/v sodium chloride, 0.06% dipotassium hydrogen phosphate) was purchased from Sigma.

2.2 METHODS

2.3 DEVELOPMENT OF POLYCLONAL ANTIBODY TO UVC-IRRADIATED DNA (UVC-DNA)

2.3.1 UVC-irradiation of polythymidylic acid [poly(dT)]

Polythymidylic acid [poly(dT)] was prepared according to the method described in Ahmad *et al.* (1999) and used as an immunogen in the production of the polyclonal antiserum IgG479. Briefly, an aqueous solution of polythymidylic acid [poly(dT)] (2mg/ml in distilled water) was irradiated using a 254nm UVC source (Knight Optical Technologies) for 2 hours on ice to prevent heating effects. Irradiation was performed at a distance of 6cm from the UVC lamp and the energy density was 21J/cm^2.

2.3.2 Immunisation protocol

The polyclonal antiserum 479 (rabbit IgG molecule) was raised against UVC-irradiated polythymidylic acid [UVC-poly(dT)] and complexed with methylated bovine serum albumin (BSA) according to the method of Plescia *et al.* (1964). Each animal was immunised according to the method described in Cooke *et al.* (1997). Briefly, reference bleeds were obtained from male, New Zealand white rabbits (n = 2) prior to immunisation. Sera were prepared by centrifugation of blood samples at 12,000 x g for 20 minutes and collection of supernatants into 1.5ml Eppendorf tubes. Collected sera were stored at -80°C. UVC-poly(dT) was made single-stranded by heat-denaturing for 10 minutes and cooled rapidly on ice. DNA solutions were then conjugated with 1% solution of methylated BSA and homogenised 1:1 with Freund's Complete Adjuvant. Animals were immunised with

1ml of immunogen, injected subcutaneously at multiple sites. Four weeks later, the animals were boosted intravenously with conjugate (DNA-BSA) alone. Test bleeds were performed 10 days later. The animals were sacrificed and the sera were collected when sufficient titre of antibody had been obtained. The latter was assessed by enzyme-linked immunosorbent assay (ELISA). The polyclonal antiserum was characterised to be specific for thymine-thymine dimers (T<>T) by competitive ELISA (Ahmad *et al.*, 1999).

2.4 Purification of polyclonal antiserum

Purification of the immunoglobulin G (IgG) fraction of the antiserum was carried out using an affinity chromatography kit (MAbTrap® G II kit), consisting of binding buffer (10 X concentrate containing 20% ethanol as a preservative), elution buffer (10 X concentrate), neutralising buffer (containing 20% ethanol as a preservative), a HiTrap Protein G column packed with 1ml of Protein G Sepharose™ High Performance, a luer adaptor and a syringe (5ml).

2.4.1 Preparation of buffers

Both binding buffer and elution buffer were prepared according to the manufacturer's instructions (Amersham-Pharmacia Biotech., St. Albans, Hertfordshire, UK.). 10 X buffer concentrate was diluted by adding 2.5ml of the 10 X binding buffer to 22.5 ml of high quality water to give a total volume of 25ml. 0.5ml of 10 X concentrate elution buffer were added to 4.5ml of high quality water to give a total volume of 5ml. Collection tubes (1.5ml Eppendorf tubes) were prepared according to the Kit's instructions; 100µl of neutralising buffer was added to 6 Eppendorf tubes, which were numbered for sequential collection of the purified fractions.

2.4.2 Purification procedure

The 5ml syringe was filled with distilled water, by removing the top cap and connecting the column and the syringe, 'drop to drop' to avoid the introduction of air into the column. The twist-off end was then removed and the ethanol preservative was washed from the column with 5ml of distilled water at a rate of ~ 1 drop per second. Following washing, the column was equilibrated by passing 3ml of diluted binding buffer through it.

Antiserum samples obtained as previously described in Section 2.3.2 were diluted with diluted binding buffer (1:1) and were then applied to the column via a syringe. The first pass of material leaving the column was collected in a bijou. Following washing of the column with 8ml of diluted binding buffer, the eluent was collected in a universal tube. Bound antibody was then eluted from the column using 5ml of elution buffer in 900µl volumes and collected in the pre-prepared tubes described above. The column was reconditioned with 5ml of binding buffer and the material collected in a bijou. After purification of antiserum, the column was equilibrated with 10ml of 20% ethanol and stored at 4°C for future use.

The absorbance for each sample was measured spectrophotometrically (Shimadzu UV-12DI) at 280 nm (A_{280} of 1 = ~0.8mg protein/ml). The fractions with the highest protein concentration were kept for future use while the rest were discarded.

2.5 Preparation of UV-irradiated DNA and synthetic oligonucleotides

Aqueous solutions of calf thymus DNA [0.5mg/ml in phosphate-buffered saline (PBS) (0.01M, pH 7.4)] were exposed to increasing doses of UVC and UVB radiation (0-8.75KJ/m^2) and 0.882KJ/m^2 of UVC radiation only in a plastic tissue culture plate. The

latter dose was used in ELISA experiments where calf thymus DNA was used as a solid phase antigen. Irradiations were performed at a distance of 6cm and 25cm from the UVC and UVB lamp, respectively (previously described in Section 2.1.3). Synthetic oligonucleotides were exposed to UVC radiation in a similar manner to the above procedure, using a dose of 14.4 KJ/m^2 and at a distance of 6cm from the UVC lamp. All irradiations were carried out on ice to prevent heating. A higher distance (25cm) from the UVB lamp was used in the UVB-irradiation of DNA samples compared to that (6cm) used in the irradiation of DNA with the UVC lamp. The different distances used in the irradiation experiments could be explained by the different UVR wavelengths as for the same dose of UV irradiation a smaller distance is required from the UVC lamp compared to the UVB lamp. Distances lower that 25cm from the UVB lamp resulted in rapid (measured in seconds) exposure of DNA samples while a distance of 25cm was found to be suitable for induction of UVB-induced T<>T in DNA samples and the exposure time was measured in minutes. Similarly, lower distances from the UVC lamp were required for the UVC-irradiation of DNA and oligonucleotides as the greater the distance the longer the exposure time. Control samples of calf thymus DNA [0.5mg/ml in PBS (0.01M, pH 7.4)] and oligonucleotides were assessed in the same manner to the UV-irradiated DNA samples but were covered with aluminium foil in order to prevent exposure to UV radiation (sham irradiated). Measurements of irradiance were performed using the UVC (MP-125) and UVB (MP-131) sensor as described earlier in Section 2.3.1, in conjunction with the optical MP-100 radiometer. The amount of DNA for each sample was determined spectrophotometrically (Shimadzu UV-12DI) at 260 nm (A_{260} of 1 = 50μg DNA/ml).

2.6 Enzyme-linked immunosorbent assay (General protocol)

96 well ELISA plates were coated with poly-L-lysine (25μg/ml in PBS; 50μl/well) and incubated at 4°C overnight in a humidified chamber. Solutions of calf thymus DNA (UV-irradiated and sham irradiated DNA) solutions were made single-stranded by heat denaturing for 10 minutes in a screw cap Eppendorf tube in a water bath followed by a rapid cooling on ice. UVC-irradiated (0.882KJ/m^2), single-stranded DNA (UV-ssDNA) solutions were used as the solid phage antigen and bound to an ELISA plate at 50μg/ml in PBS (50μl/well) by incubation at 37°C for one hour in a humidified chamber. All incubations were carried out at 37°C for one hour in a humidified chamber. Following three washes in PBS, free sites were blocked by incubation with a 4% (w/v in PBS) dried skimmed milk powder solution (200μl/well). The test antiserum (1:2000 in 4% milk/PBS) was then added to the plate (50μl/well) the wells were then washed three times with PBS containing 0.05% (v/v) Tween 20. Detection of bound antiserum was achieved using peroxidase-labelled goat anti-rabbit immunoglobulin (IgG) diluted 1:2000 (in 4% w/v dried milk/PBS; 50μl/well) following washing of wells three times with PBS containing 0.05% (v/v) Tween 20. Peroxidase-labelled antibody was detected using 50μl/well of orthophenylenediamine (0.5mg/ml in 0.05M phosphate-citrate, pH 5.0 and containing 0.03% w/v sodium perborate) as substrate solution. The reaction was stopped after 15-minute incubation at room temperature using 2M H_2SO_4 (25μl/well). The resulting absorbances were read at 492nm using a plate reader (Anthos 2001).

2.7 Competitive ELISA (General protocol)

Single-stranded, UVC-irradiated (0.882KJ/m^2) calf thymus DNA was used as the solid phase antigen and bound to a 96-well poly-L-lysine coated ELISA plate at 50μg/ml in PBS (50μl/well), after which the plate was washed three times with PBS. All incubations were

carried out at 37°C for one hour in a humidified chamber. Free sites were then blocked by incubation with a 4% (w/v in PBS) dried skimmed milk powder solution (200µl/well) and the wells were then washed with PBS. 25µl/well of serially diluted, variously treated, single-stranded solutions of the competitors in 4% w/v dried milk/PBS were applied to the plate, along with 25µl/well of the diluted polyclonal antiserum (1:5000 in 4% w/v dried milk/PBS). Following washing of wells three times with PBS containing 0.05% (v/v) Tween 20, detection of bound antiserum was achieved using peroxidase-labelled goat anti-rabbit immunoglobulin (IgG) diluted 1:2000 (in 4% w/v dried milk/PBS; 50µl/well) in conjunction with orthophenylenediamine (0.5mg/ml in 0.05M phosphate-citrate, pH 5.0, and containing 0.03% w/v sodium perborate; 50µl/well) substrate solution. The reaction was stopped using 2M H_2SO_4 (25µl/well) and the resulting absorbance was read at 492nm using a plate reader (Anthos 2001). Percentage inhibition of antibody binding to the solid phase antigen by competitive inhibition was calculated as follows:

$$\% \text{ inhibition} = \left(1 - \left(\frac{A_{492} - \text{No primary}}{\text{No competitor} - \text{No primary}} \right) \right) \text{X } 100$$

Statistical analysis was performed by GraphPad Software Prism, version 2.01, and Microsoft Excel 2000.

2.8 Determination of levels of 8-oxo-2'-deoxyguanosine in urine

2.8.1 Urine collection

First void urine samples were collected for 7 days following the first dose of treatment and continued by collection of urine samples at days 1 and 3 of week 2, day 1 of week 4 and day 3 of week 6. Upon collection, urine samples were stored, without any additives, at -80°C in 20ml plastic Universal tubes prior to analysis. Following thawing and centrifugation (300 x g for 10 min), the supernatants were analysed either by a competitive *in vitro* ELISA kit for quantitative measurement of the oxidative DNA adduct 8-oxo-2'deoxyguanosine (8-oxodG) in urine according to the manufacturer's instructions (Japan Institute for the Control of Aging; Fukuroi City, Japan) or by a competitive ELISA for thymine-thymine dimers (T<>T) as described below (Section 2.8.3).

2.8.2 8-oxodG ELISA assay

Measurements of 8-oxodG in the urine of psoriasis patients were performed using 8-oxodG ELISA kit according to the manufacturer's instructions (Japan Institute for the Control of Aging; Fukuroi City, Japan).

2.8.2.1 Assay procedure

All reagents of the 8-xodG kit were brought to room temperature prior to use. 50µl/well of standard 8-oxodG solution (0.5-200ng/ml) or urine sample were applied to the plate, along with 50µl/well of the diluted monoclonal antibody in PBS. Following tight sealing of the plate with adhesive strip, the plate was incubated at 37°C for one hour. Following washing in PBS (X 5 concentrated (v/v) diluted in distilled water; 250µl/well) by hand, 100µl/well of the diluted secondary antibody in PBS were added to the plate and incubated at 37°C for

one hour. Following washing, detection of bound secondary antibody was performed using 100µl/well of enzyme substrate solution (150µl of 3, 3', 5, 5'-tetramethylbenzidine diluted with 15mls of hydrogen peroxide/citrate-phosphate). The reaction was stopped after 15-minute incubation at room temperature in the dark using 1M phosphoric acid (100µl/well). The resulting absorbances were read at 450nm using a plate reader (Anthos 2001), after a 3-minute incubation at room temperature.

2.9 Restriction digestion of genomic DNA (General protocol)

Genomic DNA samples (both UVC- or UVB-irradiated and non-irradiated DNA) were digested by incubation with the restriction endonuclease *Bam*HI (5U/mg DNA) at 37°C overnight. All restriction digestions were performed using the reagents described below:

REACTION COMPONENTS	QUANTITY
10X React^{R} 3 buffer	2µl
DNA	1µg
Restriction enzyme	0.5µl
Nuclease-Free Water	Up to 20 µl

The reagents were added in the order listed above and mixed gently by pipetting. Aliquots of the restriction mixture were checked for completeness of digestion by agarose minigel electrophoresis.

2.10 Analysis of restriction digestion by agarose gel electrophoresis (General protocol)

Restriction digested genomic DNA samples were analysed by running an aliquot of each of the digests on a 1% agarose gel. Prior to gel casting, 1.0g of dried agarose was dissolved in 100ml of 1 X Tris-Borate electrophoresis buffer (TBE; 107.8g of Tris base, 53g of boric acid and 7.44g of disodium EDTA, dihydrate in 800 ml of distilled water; the pH was adjusted to 8.3 with boric acid and final volume was brought to 1 litre with distilled water) by heating on a hot plate till agarose was completely dissolved. The warm gel solution was then poured into a mould (made by wrapping clear tape around and extending above the edges of an 18 cm X 18 cm glass plate), which was fitted with a well-forming comb, and left to cast. Following casting of the gel, the comb was removed and the gel was placed in the electrophoresis tank along with sufficient volume of 1 X TBE buffer. 10µl of *Bam*HI-digested DNA (UV-irradiated and native DNA) samples were mixed with 2µl of gel loading buffer and loaded into the wells. The gel was run at 5 V/cm^2 for 1 hour. Following electrophoresis, the gel was stained with ethidium bromide (0.5µg/ml in distilled water) for 30 minutes at room temperature, washed with water and visualised by ultraviolet light.

2.11 PCR amplification of genomic DNA (General protocol)

Genomic DNA samples (both UVC- or UVB-irradiated, *Bam*HI digested genomic DNA) were PCR amplified using HotStartTaq DNA polymerase along with the 10 X PCR buffer [25mM Tris-HCl, 50mM KCl, 50mM $(NH_4)_2SO_4$, and 15mM $MgCl_2$; pH 8.7]. The oligonucleotide primers used in PCR amplification of genomic DNA were H-*ras*7 (sense): 5'-**AGACCTTGTAGGAGGACCCCGGGCC** and H-*ras*8 (antisense): 5'-**ATAGTGGGGTCGTATTCGTCCACAA**. PCR experiments were performed in a PCH-3 Dri-Block® Cycler using the reagents of the table below:

REACTION COMPONENTS	VOLUME	FINAL CONCENTRATION
10 X PCR Buffer	5µl	1 X diluted
dNTP mix (10mM each of dATP, dTTP, dCTP, dGTP	1µl	0.2mM (each of the dATP, dTTP, dCTP, dGTP
Upstream primer (H-*ras*7)	1µl	0.2µM
Downstream primer (H-*ras*8)	1µl	0.2µM
HotStartTaq DNA Polymerase, 5U/µl	0.5µl	2.5U/µl
Nuclease-free PCR grade water	22.75µl	
Genomic DNA, 1 mg/ml	1µl	50ng (0.05mg/ml)
Final volume	50µl	

All reagents were kept frozen at -20°C prior to use and PCR experiments were performed at room temperature. The reagents were added in the order listed above and mixed gently by pipetting. The thermal cycling profile consisted of a 15-minute heat-activation step of HotStarTaq DNA polymerase at 95°C followed by thirty cycles each one consisted of 30 sec of DNA denaturation at 95°C, 30 sec of primer-template hybridisation at 55°C, and 30 sec of primer extension at 72°C, and a final extension of 10 minutes at 72°C. PCR positive (unirradiated, *Bam*HI digested genomic DNA samples) and negative controls (no DNA just PCR reagents) were PCR amplified in the same way and included in each PCR.

2.12 Analysis and quantification of PCR products

Following amplification, the PCR products were analysed on a 2.0% agarose gel run at 70 V/cm^2 for two hours along with the 123bp DNA Ladder (3mg/lane). The gel was stained with ethidium bromide (5.0µg/ml in distilled water) and visualised by ultraviolet light. The band intensities were determined by scanning the gels with a laser scanner and integrating

the data for peak areas with computer assisted software (QuantiScan BIOSOFT^{R} software, version 2.1). Genomic DNA samples (unirradiated, *Bam*HI-digested; 0-5ng), which were PCR amplified alongside the test DNA samples and run in parallel, were used as standards in the quantification of PCR products by comparing the band intensity of the 149bp PCR product. Statistical analyses were performed using GraphPad Software Prism, version 2.01.

2.13 Real-time quantitative PCR

Real-Time PCR was performed using Brilliant® SYBR® Green Quantitative PCR Core Reagent Kit (Stratagene), the contents of which are shown in the table below:

KIT CONTENTS	QUANTITY
SureStart^{R}Taq DNA Polymerase, 5U/µl	500U
Core PCR buffer, 10 X	1.7ml
Magnesium Chloride ($MgCl_2$), 50mM	1.5ml
dNTP mix, 20mM (5mM each of dATP, dTTP, dCTP, dGTP)	400µl
DMSO, 100%	500µl
Glycerol, 50%	2.0ml
Reference dye, 1mM	100µl
SYBR® Green I stock solution 10,000 X	25µl

All reagents were kept frozen at -20°C prior to use and kept on ice during the whole experimental procedure. Dilutions of SYBR® Green I and reference dye were performed using nuclease-free PCR grade water. UVC-irradiated (1.47KJ/m^2), *Bam*HI-digested human genomic DNA (50ng per PCR) was amplified in the MX4000® Multiplex

Quantitative PCR System (Stratagene). Experimental reaction was performed using the reagents of the table below and in the order shown:

REACTION COMPONENTS	VOLUME	FINAL CONCENTRATION
Nuclease-free PCR grade water	22.75µl	
10 X Core PCR Buffer	5µl	1 X diluted
20mM $MgCl_2$	1.5µl	1.5mM
20mM dNTP mix	2µl	0.2mM (each of the dATP, dTTP, dCTP, dGTP
Upstream primer (H-*ras*7)	1µl	0.2µM
Downstream primer (H-*ras*8)	1µl	0.2µM
50% Glycerol	8µl	8%
100% DMSO	1.5µl	3%
Diluted reference dye (1:500)	0.75µl	30nM
Diluted SYBR Green I (1:2000), 10,000 X	5µl	0.5 X
SureStart^{R}Taq DNA Polymerase, 5U/µl	0.5µl	2.5U/µl
Genomic DNA, 1 mg/ml	1µl	50ng (0.05mg/ml)
Final volume	50µl	

All the PCR reagents were mixed gently by pippetting, avoiding the creation of bubbles, as they interfere with fluorescence detection. Following mixing, the samples were centrifuged briefly to collect the contents at the bottom of the tube. The thermal cycling profile consisted of a 15-minute heat-activation step of SureStart^{R}Taq DNA polymerase at 95°C followed by thirty cycles each one consisted of 30 sec of DNA denaturation at 95°C, 30

sec of primer-template hybridisation at 55°C, and 30 sec of primer extension at 72°C, and a final extension of 10 minutes at 72°C.

2.14 Immuno-Coupled Polymerase Chain Reaction (ICPCR) assay (General protocol)

Detection of DNA damage induced by different types of UV radiation (UVC and UVB radiation) in gene-specific sequences was performed in a single 0.5ml Gene Amp® Reaction tubes. 100µl of 100µg/ml of protein A in sodium bicarbonate buffer, pH 9.5, were added to each tube. After overnight incubation at 4°C, the tubes were washed twice with 150µl of PBS (0.01M, pH 7.4) followed by addition of 100µl antiserum IgG479 (1mg/ml in PBS). The tubes were incubated for 4 hrs at 4°C, washed twice with 500µl PBS and blocked with 500µl Blotto blocker (20mM Tris-HCL, pH 8.0; 120mM glycine; 5% dry milk powder; 0.5% gelatin; 0.01% thimerosal; 0.01% antifoam-A and 0.1% Tween 20) for 1hr at 37°C. Following washing with PBS, 100µl (50ng) of unirradiated or UV-irradiated, *Bam*HI-digested genomic DNA in PBS containing 0.5M NaCl was added to the tubes and incubated overnight at 4°C. Following incubation, the tubes were washed twice with 500µl PBS and once with 200µl Tris-HCL (10mM, pH 8.3).

PCR amplification of genomic DNA was conducted as described in section 2.11. Following amplification, the PCR products were analysed on a 2.0% agarose gel run at 70 V/cm^2 for two hours along with the 123bp DNA Ladder (3mg/lane). The gel was stained with ethidium bromide (5.0µg/ml in distilled water) and visualised by ultraviolet light. The band intensities were determined by scanning the gels with a laser scanner and integrating the data for peak areas with computer assisted software (QuantiScan BIOSOFT^R software, version 2.1). Genomic DNA samples (unirradiated, *Bam*HI-digested; 0-5ng), which were PCR amplified alongside the test DNA samples and run in parallel, were used as standards

in the quantification of PCR products by comparing the band intensity of the 149bp PCR product. Statistical analyses were performed using GraphPad Software Prism, version 2.01.

2.15 Gas Chromatography-Mass Spectrometry (GC-MS) analysis of cyclobutane thymine dimers

Assessment of global genome levels of T<>T was performed by stable isotope-dilution mass spectrometry, as described in Podmore *et al.* (1996). Briefly, the stable, isotopically labelled internal T<>T standard was prepared using thymine-α- α- α, 6-$^2H_4$. High performance liquid chromatography (HPLC) was used to checked the purity of T<>T, and found to be free of thymine. Following addition of the deuterated T<>T internal standard (*cis-syn* cyclobutadithymine-$^2H_8$; 2nmol was added as internal standard to each aliquot containing 100μg DNA), aliquots of the treated DNA were subjected to formic acid hydrolysis (60% v/v) prior to derivatisation with N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA)/acetonitrile; 4:1 v/v). GC-MS in selected ion monitoring (SIM) mode was performed using a Perkin-Elmer Autosystem gas chromatograph coupled with a Perkin-Elmer Q-mass 910 quadrupole mass spectrometer in selected ion monitoring mode.

2.16 Cell culture protocol

Spontaneously immortalised human keratinocyte (HaCaT) cells were grown to confluence (90-100% confluence) in 300cm^2 tissue culture flasks in DMEM medium at 37 °C under a humidified atmosphere of 95% air and 5% CO_2.

Routine subcultures were obtained by washing the cells in Hank's Balanced Salt Solution (HBSS) and trypsinising them with HBSS containing 0.1% trypsin (15ml of HBSS/Trypsin solution per culture flask). Following passage/trypsinisation of cells, 20ml of warm DMEM media were added to the cells in order to neutralise the effects of trypsin and the cells were pelleted by centrifugation at 1200 rpm for about 5 minutes. The cell pellets were then resuspended in 5mls of warm DMEM media and incubated (1:4 dilution) in culture flasks containing DMEM media at 37 °C. Cells were fed and passaged on a rotational basis, half the flasks were trypsinised and given fresh media one day and the remainder were trypsinised the following day, in order to prevent contamination of the cells. At various passage numbers cells were frozen in cell freezing medium (DMSO) and stored in freezing vials in liquid nitrogen.

2.16.1 Cell counting

Cell numbers were determined by staining with Trypan Blue dye. The cell pellets obtained from trypsinisation of cells were resuspended in 5ml of media (DMEM). A cell suspension (20µl) was then mixed by pipetting with an equal volume of Trypan Blue stain and allowed to stand for 5 minutes at room temperature, in order for trypan blue to penetrate non-viable cells that might be present in the cell suspension. The solution was then placed in the two chambers of a haemocytometer by carefully touching the edge of the cover-slip with the pipette tip and allowing each chamber to fill by capillary action. Viable cells (non-viable cells stained blue and viable cells remained white) were then counted in the 4 outside corners and the middle square in both chambers under x20 microscope magnification. The total number of cells was determined using the following calculations:

Viable cells per ml = viable cells counted per square x 10^4 x dilution factor

Total cells = cells per ml x original volume (resuspension volume of media)

where, 10^4 is the standard number for converting the volume of cells counted in the haemocytometer to 1ml.

2.17 UVB-irradiation of human keratinocyte cells

HaCaT cells were cultured as described above (Section 2.16.1) in 300cm^2 tissue culture flasks. At 80-90% confluence, cells were seeded into seven 100mm Petri dishes at a density of 2.9 x 10^6 cells and incubated overnight at 37°C. Following incubation, culture medium was removed and cells were washed twice with HBSS (5mls per Petri dish), after which they were irradiated on ice in PBS buffer for a dose range of 0-1KJ/m^2 of UVB radiation. Each dish represented a single UVB dose. Irradiations were performed at a distance of 22cm from the UVB lamp previously described in Materials and Methods Section and the irradiance was measured using the UVB (MP-131) sensor, in conjunction with the optical radiometer (MP-100). Throughout the irradiation of cells, the output of the UVB lamp was continually monitored by the MP-100 radiometer in order to ensure that the intended UVB doses were delivered to the cells. Following irradiation or after a repair time of 0-24 hours, the cells were washed with PBS buffer and either collected immediately following trypsinisation or further incubated in serum-free DMEM medium at 37°C to allow DNA repair to occur. Cell pellets were processed immediately for isolation of DNA using Wizard® Genomic DNA Purification kit.

Following a period of repair (0-24 hours), supernatants from cell culture medium were collected in 1.5ml microcentrifuge tubes and assayed by competitive ELISA for the

presence of UVB-induced cylobutane thymine dimers in cell media. Neat culture medium was used as a negative control and medium from cells not exposed to UVB radiation was used as a positive control.

2.18 Isolation of genomic DNA from cells

Isolation of genomic DNA from human cultured HaCaT cells was carried out using the Wizard® Genomic DNA Purification kit according to the manufacturer's instructions (PROMEGA, Southampton, U.K.). Cell pellets were transferred to a sterile 1.5ml microcentrifuge tube and resuspended in 1.0ml PBS by pipetting. Following centrifugation at 13000 x g for 15 seconds and careful removal of supernatant, cells were lysed in 600µl of Nucleic Lysis Solution by pipetting until no visible cell clumps remained. 3µl of RNase Solution was then added to the nuclear lysates and mixed by inverting the tubes 25 times. The mixtures were then incubated for 30 minutes at 37°C. Following incubation, samples were allowed to cool for 5 minutes at room temperature and 200µl of Protein Precipitation Solution was added with vigorous vortexing for 20 seconds. The samples were then chilled on ice for 5 minutes prior to centrifugation at 13000 x g for 4 minutes. A tight white protein pellet was visible at that stage. The supernatant containing the DNA (leaving the protein pellet behind) was then carefully removed and transferred to a sterile 1.5ml microcentrifuge tube containing 600µl of room temperature isopropanol. The solution was then gently mixed by inversion until the white thread-like strands of DNA formed a visible mass and centrifuged at 13000 x g for 1 minute at room temperature. The supernatant was carefully discarded leaving only genomic DNA in the tube, which was visible as a small white pellet. Finally, the DNA was washed in 600µl of room temperature ethanol (70%) by gently inverting the tubes several times and centrifuged at 13,000g for 1 minute at room temperature. Ethanol was aspirated using a Pasteur pipette and care was taken in order to

avoid aspiration of DNA into the pipette. DNA pellets were then allowed to air dry for 10-15 minutes at room temperature and were rehydrated in 100µl of DNA Rehydration Solution (10mM Tris-HCL, pH 7.5, 1mM EDTA) by overnight incubation at room temperature. The amount of DNA for each sample was determined spectrophotometrically (Shimadzu UV-12DI) at 260nm (A_{260} of 1 = 50µg DNA/ml).

2.19 Measurement of cell viability by MTT assay

Viability of HaCaT cells in response to UVB radiation was determined using the colorimetric MMT assay previously described by Mosmann (1983). MTT [3-(4,5-cimethylthiazol-2-γl)-2,5-diphenyl tetrazolium bromide is a pale yellow substrate that is cleaved by viable cells to yield purple formazan crystals. Cells were seeded (70-80% confluent) into 6-well culture plates at a density of 2.5 x 10^5 cells per well and allow to adhere overnight in growth DMEM medium. Following overnight incubation at 37°C, culture medium was removed and the cells were washed once with HBSS (1ml per well). Cells were irradiated uncovered on ice in HBSS buffer for a dose range of 0-1KJ/m^2 using the UVB lamp previously described. Following irradiation, cells were washed twice in HBSS buffer and incubated in serum-free medium for appropriate post-treatment times (0-24 hours) to detect the effects of UVB radiation on the viability of cells. Control, untreated cells were included in each experiment. Following incubation, cells washed twice in HBSS buffer and 100µl of diluted MTT (0.5mg/ml in DMEM without serum, which was filtered through a syringe to sterilise) was added to each of the wells and incubated for 4 hours at 37°C. After MTT incubation, cell supernatants were removed and the cells were washed once with HBSS buffer. Following washing, 1ml of DMSO was added to the cells by incubation for 5 minutes on a rotating platform to mix at room temperature. The DMSO dissolves the formazan to yield a homogenous dark purple solution suitable for absorbance

measurement. Absorbance was read at 570nm using Shimadzu UV-1201 spectrophotometer and the results were expressed as percentage of the control, unirradiated cells. Statistical analysis was performed using Student's *t*-test provided as part of GraphPad Prism Software (version 2.01).

2.20 Indirect immunofluorescence microscopy

HaCaT cells were seeded in eight-well chamber slides (5 x 10^4 cells per well) and allow to adhere overnight in DMEM medium at 37°C. Following overnight incubation, culture medium was carefully removed and cells were washed once with PBS (0.01M, pH 7.4) buffer. Cells were irradiated in HBSS buffer at 0.2 and 0.8KJ/m^2 of UVB radiation using the UVB lamp previously described (Section 2.1.3). Control, unirradiated cells were analysed in the same way and included in each experiment. Following irradiation, the cells were washed twice with PBS buffer and fixed with 100% cold ethanol (200µl per well) for 5 minutes at room temperature. Excess ethanol was discarded by pipetting and cells were allowed to dry at room temperature. Cells were then blocked with 2% normal goat serum (NGS) in PBS (200µl per well) and incubated at 37°C for thirty minutes. Following blocking with serum and washing three times with PBS, antiserum IgG479 (1:7500 in NGS/PBS; 200µl per well) was added to the cells and incubated at 37°C for one hour. Following three washes with PBS, FITC-labelled goat anti-rabbit secondary antibody, (1:320 in NGS/PBS; 200µl per well; 492nm excitation wavelength and 515nm emission wavelength) was added to the wells by incubation at 37°C for one hour in the dark. All procedures from this point onwards were performed in the dark. The cells were then washed three times with PBS and incubated with DAPI (1µg/ml in PBS; 200µl per well) for five minutes at room temperature. Following washing of cells three times with PBS, slides were mounted gently with Vectashield fluorescence mounting medium (2 drops in

the two corners of the slide) and immunostained cells were viewed by Zeiss Axioskop fluorescence microscope at x 400 magnification.

CHAPTER 3: IMMUNOCHEMICAL DETECTION OF UVR-INDUCED DAMAGE IN DNA

CHAPTER 3

IMMUNOCHEMICAL DETECTION OF UVR-INDUCED DAMAGE IN DNA

3.1 INTRODUCTION

Several methods have been developed for the detection and quantification of ultraviolet radiation (UVR)-induced dimer damage to DNA. Southern hybridisation-based assay was used in the quantification of cyclobutane pyrimidine dimers (CPDs) employing lesion-specific enzymes, such as T4 endonuclease V (Seawell *et al.*, 1980), and UV-endonuclease from *Micrococcus luteus* (Sutherland and Shih, 1983) by electrophoresis in alkaline agarose gels. The ^{32}P-postlabeling assay was also used for the measurement of CPD (Bycov *et al.*, 1995) and other lesions in DNA including oxidative DNA damage (Phillips, 1997). High-performance liquid chromatography (HPCL) is another technique that has been applied to the quantification of CPD, particular cyclobutane thymine dimer (T<>T), within isolated and radiolabeled cellular DNA (Cadet *et al.*, 1983) following formic acid treatment. Gas-chromatography (GC-MS) has been used in the measurement of *cis-syn* T<>T (Podmore *et al.*, 1996) within isolated DNA. In addition, the formation of photoproducts at the sequence level in cellular DNA has been monitored by ligation-mediated polymerase chain reaction (LMPCR) (Pfeifer *et al.*, 1991; 1993; Drouin and Therrien, 1997) and, most recently, HPLC with electrospray ionisation tandem mass spectrometry (HPLC-MS/MS) allowed the detection of pyrimidine photoproducts within DNA (Douki *et al.*, 2000; 2003). Though important, all these techniques possess limitations, including the high cost for consumables and equipment and the requirement of extensive sample preparation prior to analysis, which is often associated with a potential

risk of artefact generation. Nevertheless these techniques remain powerful in the assessment of lesion damage to DNA and will continue to be used as a reference to compare data obtained from alternative approaches.

The immunological detection of DNA lesions provides an alternative to the chromatographic and enzymatic methods used to measure UVR-induced lesions in DNA. Their ability to localise DNA lesions within a sample and the minimal risks associated by their use make immunological methods suitable for the detection and quantification of modified DNA bases within individual cells (Cooke *et al.*, 2000b) or tissues (Young *et al.*, 2000) as well as in serum (Cooke *et al.*, 1998), cell culture supernatants (Kantha *et al.*, 1996) and urine (Cooke *et al.*, 2001). Following the initial development of a polyclonal antiserum against UV-irradiated DNA (Levine *et al.*, 1966), polyclonal and monoclonal antibodies raised against numerous forms of base damage, such as T<>T and (6-4) photoproducts (Muller and Rajewsky, 1980; Mori *et al.*, 1991; Blount *et al.*, 1990; Herbert *et al.*, 1994), alkylated guanine residues (Prevost *et al.*, 1990), 8-oxodeoxyguanosine (Kasai and Nishimura, 1986; Park *et al.*, 1992) have been characterised allowing detection and quantification of damage in DNA.

3.2 AIM

The aim of the work presented in this chapter was (i) to fully characterise the ability of the polyclonal antiserum raised to UVC-poly(dT) to detect sequence-specific modifications induced by UVC-irradiation of DNA and (ii) detect its ability to recognise lesions in DNA after exposure to UVB radiation.

3.3 METHODS

3.1 Enzyme-linked immunosorbent assay

ELISA assay was performed in a manner similar to that described in the Materials and Methods chapter (Section 2.6), using single-stranded, UVC-irradiated (0.882KJ/m^2) calf thymus DNA as the solid phage antigen and polyclonal antiserum IgG479 as the primary antibody. Detection of primary antibody was achieved as previously described (Section 2.6) and the resulting absorbance was read at 492nm using Anthos 2001 plate reader. Analysis was performed using GraphPad Software Prism, version 2.01.

3.2 Competitive ELISA

Competitive ELISA was performed as previously described (Section 2.7) using single-stranded, UVC (0.882KJ/m^2)-DNA as the solid phase antigen. Serially diluted solutions of calf thymus DNA [0.5mg/ml in phosphate-buffered saline (0.01M, pH 7.4)] irradiated with increasing doses of UVC and UVB radiation (0-8.75KJ/m^2) (previously described in Section 2.5) and UVC-irradiated (14.4 KJ/m^2) synthetic oligonucleotides were used as competitors (diluted in 4% w/v dried milk/PBS; 25µl/well) along with 25µl/well of the diluted IgG479 (1:5000 in 4% w/v dried milk/PBS). Detection of primary antiserum was achieved as previously described (Section 27) and the resulting absorbance was read at 492nm using a plate reader (Anthos 2001, Anthos Labtec Instruments). The results were expressed as percentage inhibition and statistical analysis was performed by GraphPad Software Prism, version 2.01, and Microsoft Excel 2000.

3.4 RESULTS

3.4.1 Purification of polyclonal antiserum using MAbTrap^R G II kit.

Purification of polyclonal antiserum raised against UVC-poly(dT) was performed using MAbTrap^R G II affinity chromatography kit (previously described in Sections 2.4-2.4.2). The absorbance values of the eluted fractions were measured at 280nm and plotted for each sample using GraphPad Software Prism, version 2.01 (Figures 3.1 and 3.2). The highest absorbance value was detected in fraction 2 for both antiserum aliquots (A_{280} = 2.855nm and 2.613nm for purified fractions 1 and 2 respectively) suggesting that at these two fractions the IgG content of the antiserum was maximum. The protein concentration for the two eluted fractions with the highest absorbance value was calculated using 1 OD unit = ~0.8mg protein/ml and found to be 2.284mg/ml and 2.09mg/ml for fractions 1 and 2 respectively. Both aliquots of polyclonal antiserum were used in the experiments carried out in this study.

Purification results showed that MAbTrap^R G II kit was effective in the purification of the polyclonal antiserum as high protein concentrations were seen for both the purified IgG fractions of the serum.

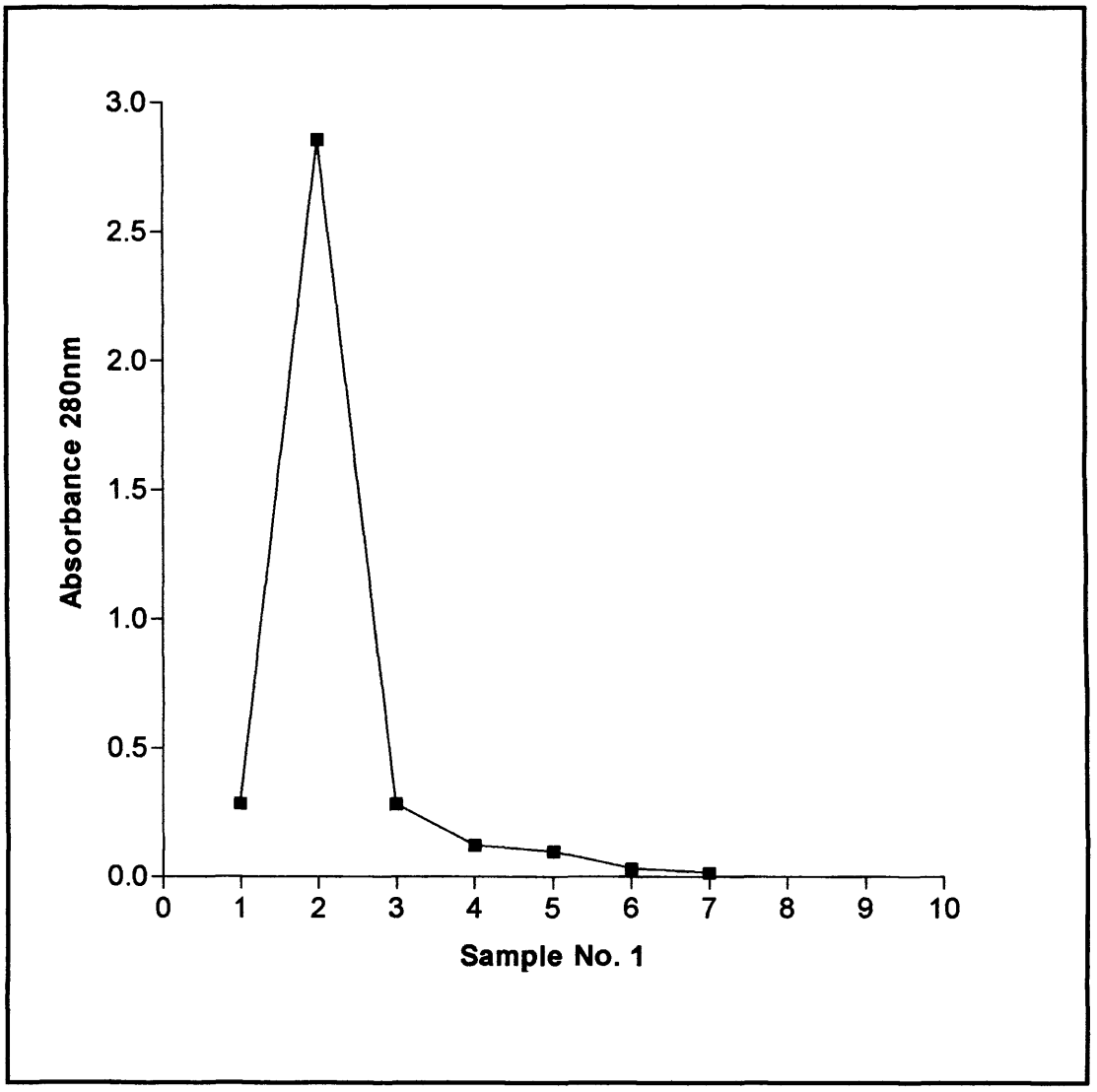


Figure 3.1: Absorbance at 280nm of eluted fractions derived from the purification of aliquot 1 of the polyclonal antiserum IgG479 using MabTrap^{R}G II kit.

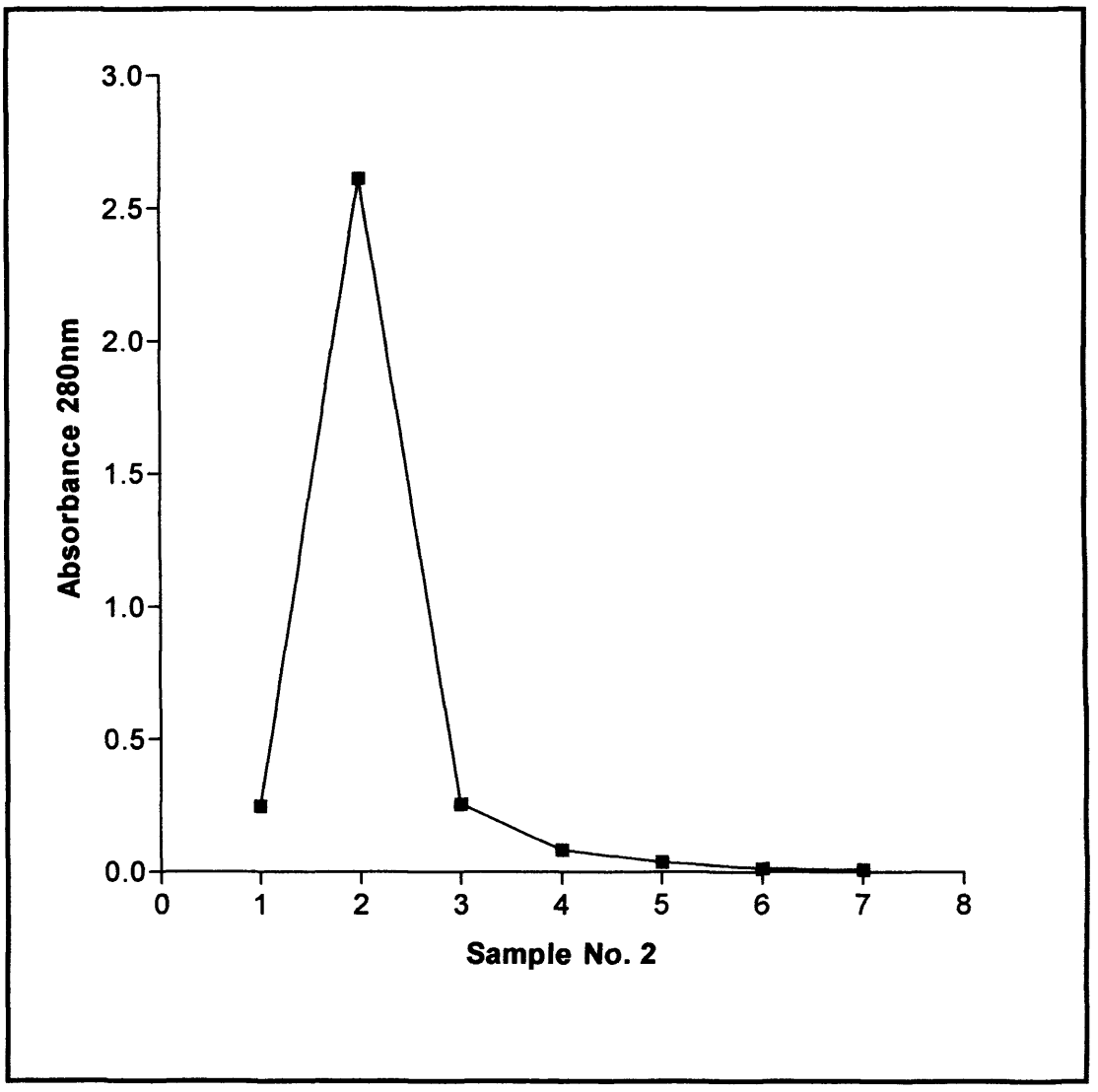


Figure 3.2: Absorbance at 280nm of eluted fractions derived from the purification of aliquot 2 of the polyclonal antiserum IgG479 using MabTrap^{R}G II kit.

3.5 Investigation of the antiserum raised against UVC-poly(dT)

Following booster injections with UVC-poly(dT)/BSA conjugate a strong immunogenic response to UVC-poly(dT) was observed such that at 1:5000 dilution of serum the absorbance at 492 nm was 1.0 whilst the binding of pre-immune serum was negligible as previously described by Ahmad *et al.* (1999). Subsequently it was demonstrated by ELISA, using an anti-rabbit IgG secondary antibody, that a large IgG response had been achieved (Ahmad *et al.*, 1999). Detection of binding of polyclonal antiserum IgG479 to UVC-single-stranded DNA was performed using an ELISA assay as described earlier. Polyclonal antiserum IgG479 was found to be specific to UVC-ssDNA as a strong immunogenic response, with an absorbance value at 492nm of 1.752 compared to 0.282 for native single-stranded DNA (Figure 3.3).

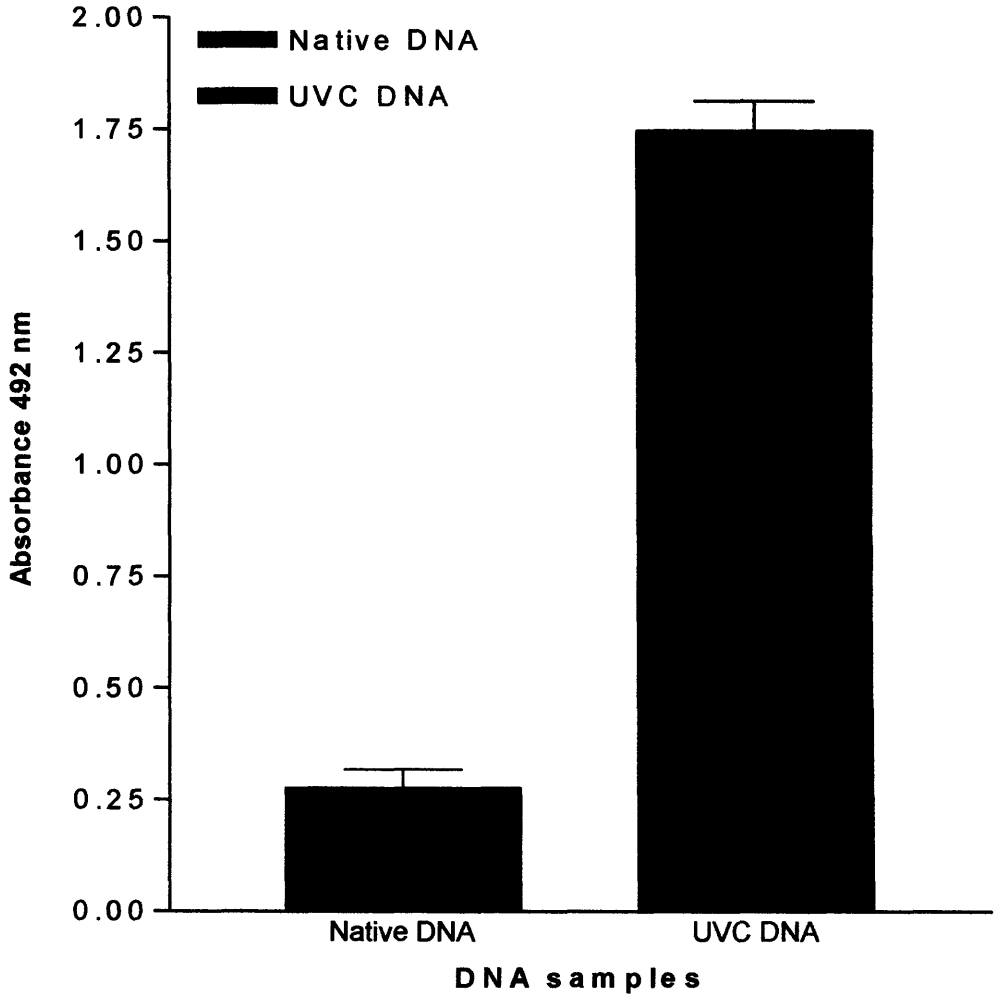


Figure 3.3: Direct ELISA showing binding of polyclonal antiserum IgG479 to UVC-irradiated and native DNA. Values represent the mean (± SEM) of three absorbances per DNA sample at 492nm.

Characterisation of the epitopes on UVC-DNA that are recognised by the antiserum was established using a competitive ELISA previously described. The antigenic specificity of the polyclonal antiserum was investigated by comparing the competitive ability of putative antigens for binding with the polyclonal antiserum. Using UVC-irradiated (0.882KJ/m^2) DNA as the solid phase antigen and a working antiserum dilution of 1:5000, UVC-double-stranded DNA (UVC-dsDNA) was found to be an effective inhibitor with an IC_{50} of 0.618μg/ml (Figure 3.4). The IC_{50} is the concentration of antigen giving 50% inhibition, providing an index of the antigenicity of the compound, and the lower the value the better the inhibitor. However, the effectiveness of inhibition increased by using UVC-ssDNA as a competitor, which resulted in an IC_{50} of 0.205μg/ml (Figure 3.4). No competition for antiserum binding was detected using native (non-irradiated) DNA up to a concentration of 100μg/ml, irrespective of whether single- or double-stranded, indicating the specificity of antiserum to recognise damage in DNA following exposure to UVC radiation.

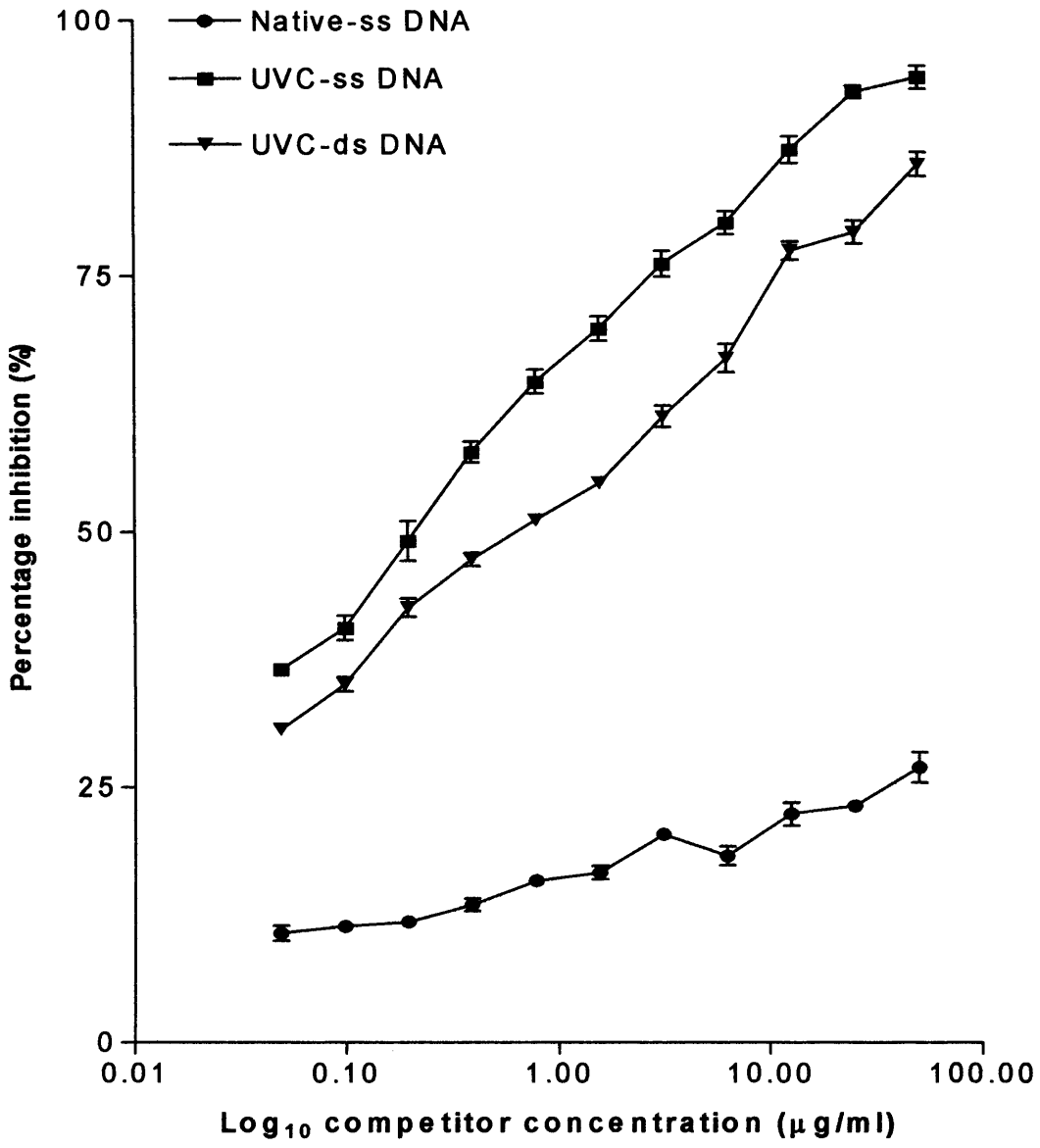


Figure 3.4: Inhibition of ELISA binding of polyclonal antiserum IgG479 to UVC-ssDNA and UVC-dsDNA. Maximum inhibition (100%) was observed in the absence of solid-phase antigen (UVC-DNA). Values represent the mean (± SEM) of three determinations per concentration.

3.6 Inhibition by UVC-irradiated polymers of thymine and sequence specificity

A series of oligonucleotides containing thymine residues was used in a competitive ELISA in order to identify the epitopes recognised by the antiserum. Polymers of thymine nucleotides were studied since it has been previously reported that pyrimidine dimers could be responsible for the immunogenicity of UV-DNA (Seaman *et al.*, 1972; Herbert *et al.*, 1994).

In competitive ELISA, inhibition of antiserum binding to solid phase UVC-DNA was related to the length of UVC-irradiated oligonucleotide chain, which contained solely thymidylate residues (Table 3.1). Poly (T_2) was the least effective inhibitor with an IC_{50} of 4.114μg/ml, but with increasing chain length the effectiveness of inhibition increased, being most apparent between poly (T_2) and poly (T_{10}), which showed the greatest inhibition with an IC_{50} of 0.66μg/ml (Figure 3.5). Unirradiated thymidylate oligonucleotides were found to be extremely poor inhibitors (IC_{50}>50μg/ml). Furthermore poly(A12), poly(C12) and poly(G12) whether UVC-irradiated or not, were found to be extremely poor inhibitors (IC_{50}>100μg/ml) as shown by Ahmad *et al.* (1999), suggesting the presence of thymines as necessary for inhibition.

An extra group of oligonucleotides was used in order to investigate whether the epitope recognised by the antiserum was larger than single thymine dimers. An oligonucleotide containing alternating A and T residues (TATATATA), UVC- irradiated and native, showed no inhibition (IC_{50}>50 μg/ml) in the competition ELISA (Table 3.1; Figure 3.6), suggesting that two adjacent thymines, or at least pyrimidines, to be necessary for the binding of the antiserum. In addition, the UVC-irradiated oligonucleotide of alternating T-T and A-A residues was found to be a poor inhibitor with an IC_{50} of 13.25μg/ml (Table

3.1; Figure 3.6), indicating that dimerisation of thymine alone was not adequate in producing efficient inhibition of the antiserum. However, a strong inhibitory response was observed using a run of three thymine residues in sequence flanked by adenine residues in the UVC-irradiated AATTTAA oligonucleotide (IC_{50} of 2.167μg/ml; Figure 3.6), suggesting that at least three adjacent thymine residues to be the minimum antigen. An increase in inhibition was observed using four thymines in sequence flanked by adenine residues (IC_{50} of 1.404μg/ml; Figure 3.6).

Using UVC-irradiated oligonucleotides with a 5' and a 3' cytosine, the possibility of whether cytosines could replace thymines within the epitope was investigated. UVC-irradiated AATTCAA oligonucleotide showed a good inhibition of binding of the antiserum to UVC-DNA having an IC_{50} of 2.5μg/ml quite close with that observed using AATTTAA, suggesting that there is a preference of a 3' cytosine (Figure 3.7). The oligonucleotide with the sequence AACTTAA was found to be a poor inhibitor (Figure 3.7) with an IC_{50} of 4.3μg/ml. These results showed that inhibition increased according to the location of the third pyrimidine relative to the dimer (TTT>TTC>CTT), with TTT having an IC_{50} about two times lower than CTT. Furthermore, the oligonucleotide with a cytosine residue between two thymines, flanked by two adenines at each end (AATCTAA) was a poor inhibitor having an IC_{50} about four times higher than TTC (Table 3.1), indicating that the epitope must be a minimum of two adjacent thymines forming a dimer with a 3'pyrimidine (T or C) to elicit an optimal immunogenic response. UVC-induced cytosine-thymine (C<>T) dimers appeared, therefore, to be less important epitopes for this antiserum.

TABLE 3.1: Summary of competitive inhibition by a variety of UVC-irradiated oligonucleotides using polyclonal antiserum IgG479. Unirradiated oligonuleotides did not compete (IC_{50} >50μg/ml).

Inhibitor	IC_{50} (μg/ml)
UVC-ssDNA	0.205
UVC-dsDNA	0.618
Unirradiated DNA	>100
T2	4.114
T4	2.634
T10	0.664
TATATATA	>50
TTAATTAA	13.25
AATTTAA	2.167
AATTTTAA	1.404
AATCTAA	8.285
AATTCAA	2.525
AACTTAA	4.277

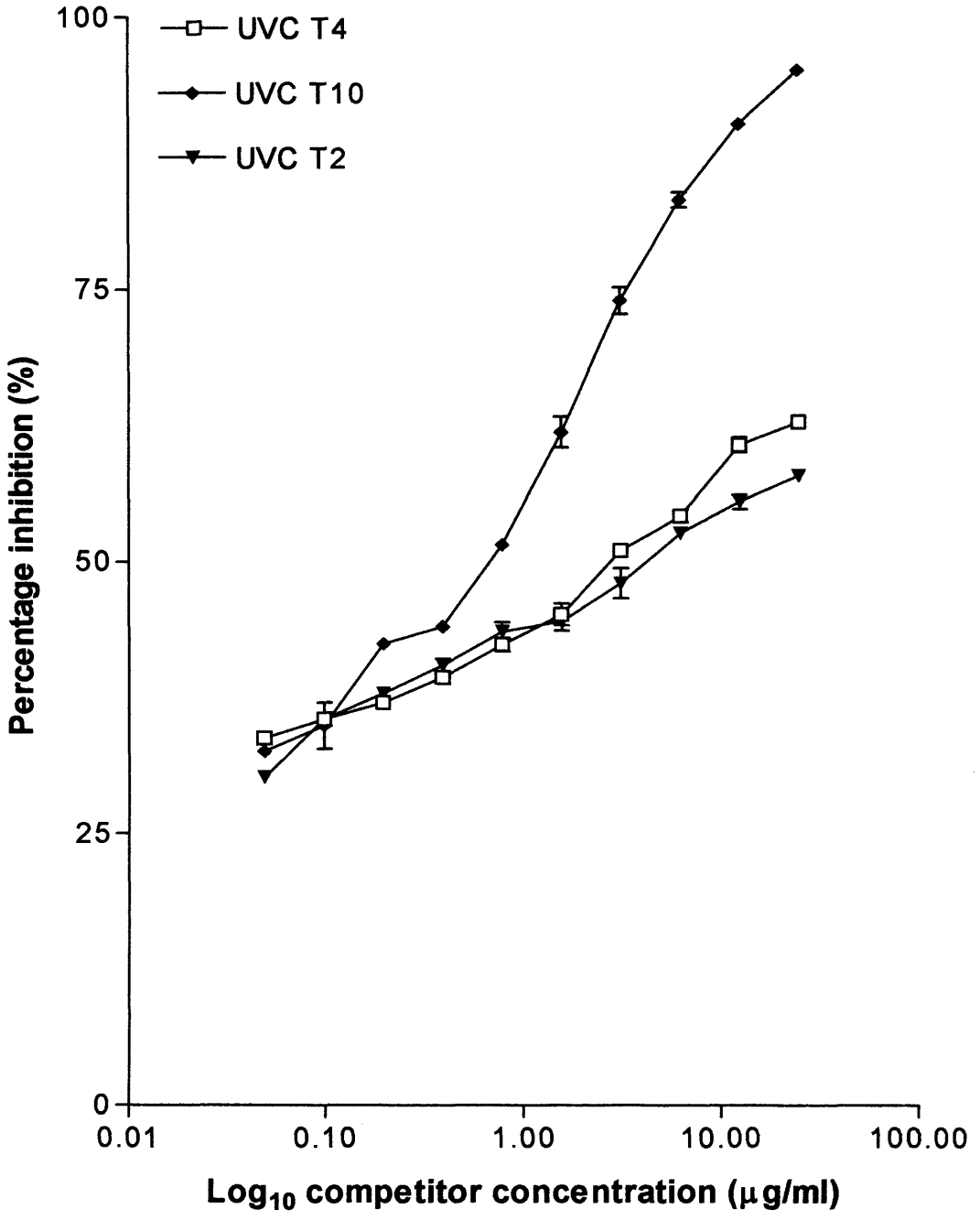


Figure 3.5: Inhibition of ELISA binding of IgG479 serum to UVC-DNA by different UVC-irradiated oligonucleotides of increasing length. Maximum inhibition (100%) was that observed in the absence of solid-phase antigen (UVC-DNA). Values represent the mean (± SEM) of three determinations per concentration.

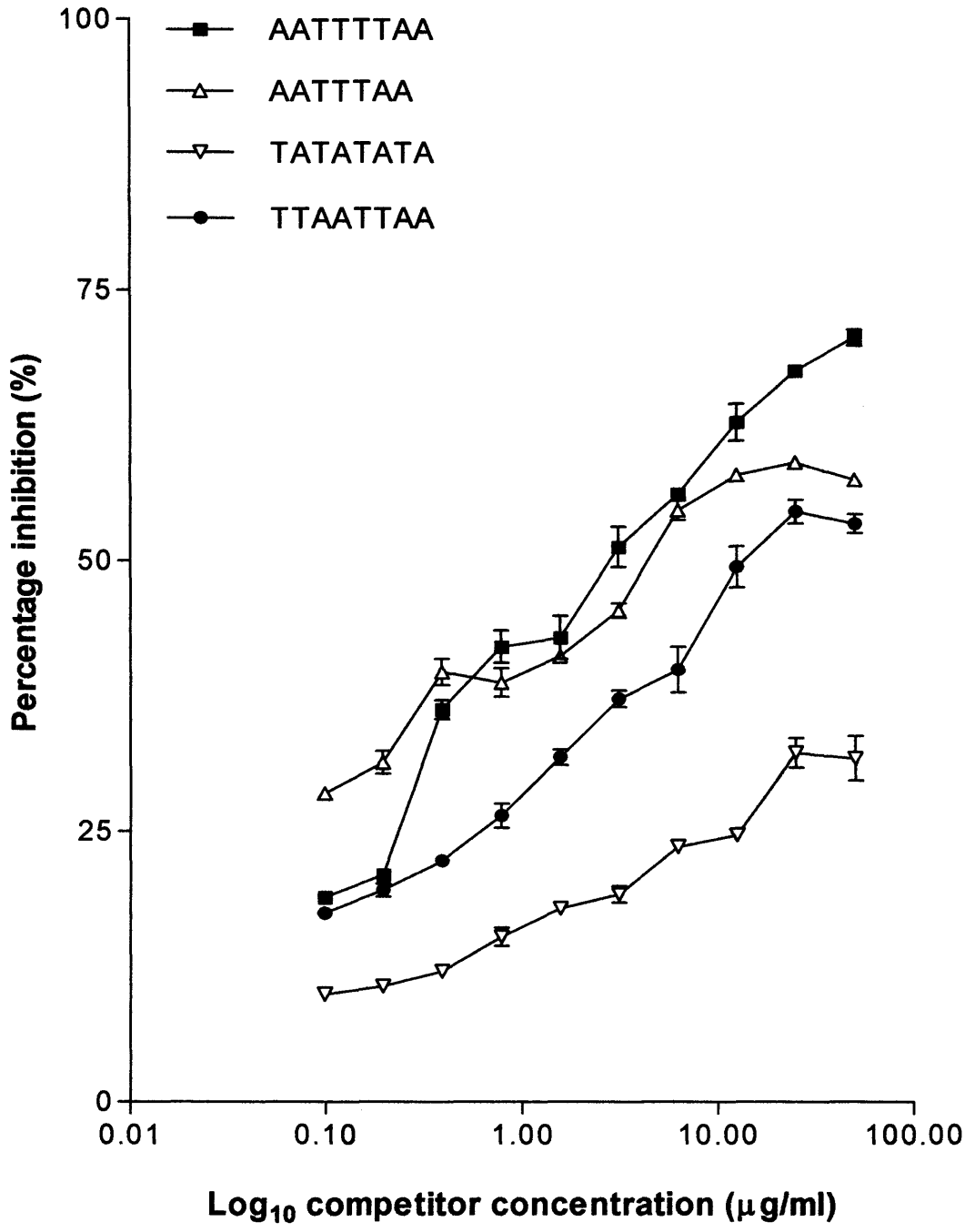


Figure 3.6: Inhibition of ELISA binding of IgG479 antiserum to UVC-DNA by different UVC-irradiated oligonucleotides of different sequence. Maximum inhibition (100%) was that observed in the absence of solid-phase antigen (UVC-DNA). Values represent the mean (± SEM) of three determinations per concentration.

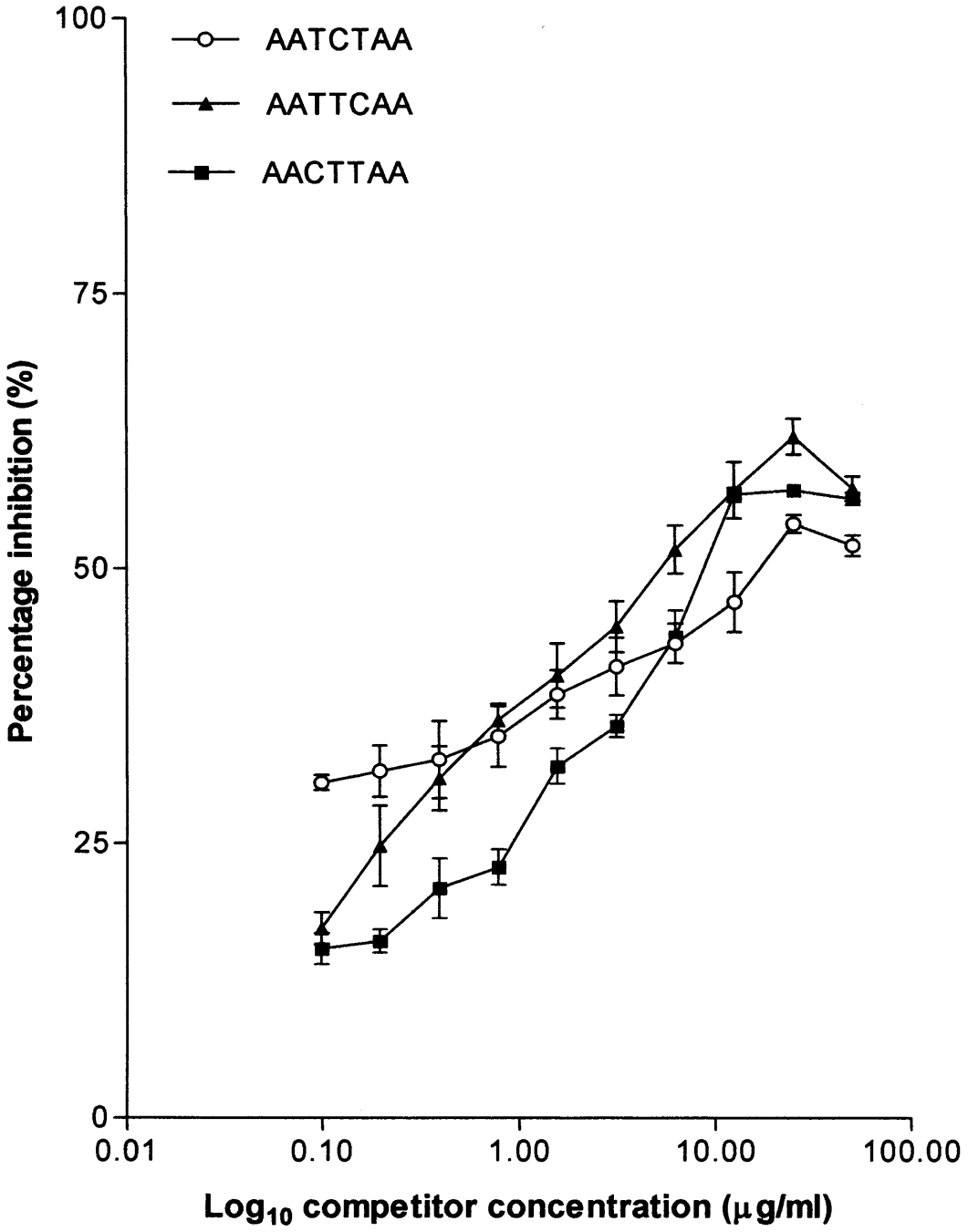


Figure 3.7: Inhibition of ELISA binding of IgG479 serum to UVC-DNA by different UVC-irradiated oligonucleotides of different sequence. Maximum inhibition (100%) was that observed in the absence of solid-phase antigen (UVC-DNA). Values represent the mean (± SEM) of three determinations per concentration.

3.7 Investigation of UVR-induced DNA damage by competitive ELISA

The polyclonal antiserum 479 (rabbit IgG molecule) raised against UVC- irradiated poly(dT) was found to specific for recognising thymine dimers (Ahmad *et al.*, 1999). The ability of this antiserum to detect UVC- and UVB-induced DNA damage was assessed using a competitive ELISA as described earlier. DNA samples were exposed to increasing doses (0-8.75 KJ/m^2) of UVC- and UVB-radiation. The dose range for UVC radiation was previously used in the GC-MS assay described by Podmore *et al.* (1996), in order to measure levels of *cis-syn* cyclobutane thymine dimers produced within DNA. Results obtained from competition ELISA showed UVC-DNA to be a better inhibitor with an IC_{50} of 0.35μg/ml compared with that of UVB-DNA (IC_{50} of 0.63μg/ml). This preferential binding of antiserum to UVC-irradiated DNA was probably due to a higher rate of T<>T induction following UVC radiation rather than UVB. However, the binding of the antiserum for the two types of radiation (UVC and UVB) reached a level of saturation at the highest dose ($8.75KJ/m^2$). No competition to native, non-irradiated DNA was observed confirming the specificity of the antiserum to UVC- and UVB-induced T<>T in DNA.

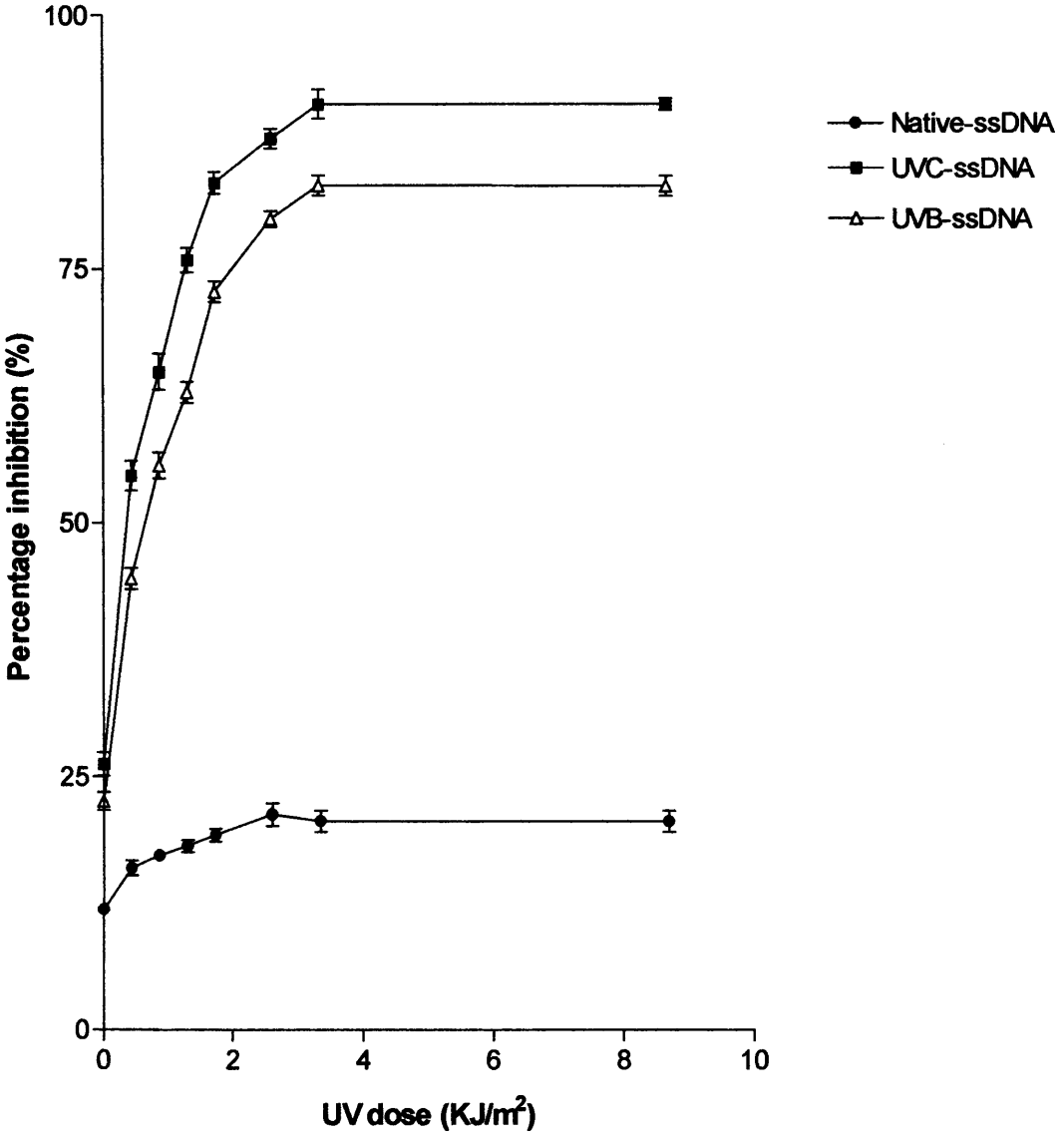


Figure 3.8: Inhibition of ELISA binding of polyclonal antiserum IgG479 to DNA following increasing doses of UVC and UVB radiation. Maximum inhibition (100%) was observed in the absence of solid-phase antigen (UVC-DNA). Values represent the mean (± SEM) of three determinations per concentration.

3.8 DISCUSSION

The ultraviolet region of the solar spectrum is the primary aetiological agent in the induction of skin cancer due to its ability to damage DNA resulting in the formation of UVR-induced DNA lesions, which can lead to permanent mutations in the DNA sequence if not repaired. In this study, the ability of polyclonal antiserum IgG479 to detect sequence-specific DNA modifications induced by UVC radiation of DNA was investigated, thereby, extending the findings of Ahmad *et al.* (1999).

Results obtained from competition experiments showed single-stranded UVC-DNA to be a better inhibitor compared to double-stranded. This observation agreed with previous studies on the effectiveness of competition of UVC-ssDNA by rabbit anti UV-DNA sera (Wakizaka and Okuhara, 1979; Strickland and Boyle, 1981, Herbert *et al.*, 1994). This preferential binding of antiserum to single-stranded DNA following exposure to UVC radiation could be due to the location of antigenic determinants of UV-DNA on exposed single-stranded regions of DNA, which have been adequately distorted by photoproducts to induce local denaturation (Wakizaka and Okuhara, 1979). UVC-irradiated oligonucleotides containing solely thymine residues, showed strong inhibition of antibody binding in competition experiments, as was originally observed by Levine *et al.* (1966). This effectiveness in the inhibition of antibody binding with increasing the length of the oligonucleotide chain is in agreement with previous studies on the characterisation of antisera using oligonucleotides containing thymine residues of increasing length (Levine et al., 1966; Strickland and Boyle, 1981; Herbert *et al.*, 1994; Ahmad *et al.*, 1999). No inhibition was demonstrated using UVC-irradiated TATATATA oligonucleotide, although UVC-induced dimerisation between non-adjacent thymines has been described (Love and Minton, 1992). A minimum of three adjacent thymine residues was essential for effective

inhibition, suggesting that conformational changes to the DNA polymer could have been conferred by the dimer and this could account for the increased immunogenicity (Cadet *et al.*, 1985). This finding agreed with previous studies on antisera raised against UVC-DNA using oligonucleotides with thymine residues (Herbert *et al.*, 1994).

Using UVC-irradiated oligonucleotides with a 5' and a 3' cytosine in a competitive ELISA, cytosines were found to be able to partially replace thymines within the smallest effective epitope 5' or 3' to T<>T, but not as a C<>T dimer. Thus, inhibition was increased according to the location of the third pyrimidine relative to the dimer (TTT>TTC>CTT), with TTT having an IC_{50} about two times lower than CTT. These results are in agreement with those obtained from the characterisation of the sequence-specificities of a polyclonal antiserum raised against UVC- and UVA-irradiated DNA (Herbert *et al.*, 1994). In addition, Umlas *et al.* (1985) showed that the ratio for the induction of T<>T and T<>C/C<>T dimers in defined DNA sequences was about 2:1.

The ability of the antiserum to bind UVR-induced DNA damage was further investigated using competitive ELISA. The results showed the induction of T<>T in DNA irradiated either with UVC or UVB. The binding of the antiserum both to UVC- and UVB-DNA increased in a dose-responsive manner over the lowest doses (0-3.48KJ/m^2) as the higher the dose of UV radiation (UVC and UVB) the higher the levels of antibody's binding. However, saturation in the binding of the antiserum for both types of UV radiation was seen, probably due to steric hindrance in the competitive ELISA, which prevents the ability of the antiserum to access other sites within the modified antigen, or because all the potential sites for thymine lesions had been identified. These results agreed with those obtained from the assessment of DNA damage after exposure to UVC and UVB radiation

by polyclonal antiserum 529, where the induction of T<>T levels reached a plateau level at the highest dose (0.875mJ/m^2) for both types of UV radiation (Cooke *et al.*, 2003b).

The UVC region (200 to 280 nm) of the electromagnetic spectrum is effectively blocked from reaching the earth's surface through being absorbed by atmospheric ozone layer (100 to 320 nm) (Gasparro and Brown, 2000). On the contrary, UVB (280 to 320 nm) is of environmental significance as it reaches the earth's surface in amounts sufficient to have important biological consequences from exposure of the skin and eyes (Ananthswamy and Pierceall, 1990). UVA radiation (320-400mn) constitutes the predominant component of solar UV radiation to which everyone is exposed. However, it appears to be less cytotoxic than UVB light as it is poorly absorbed by DNA (Sutherland and Griffin, 1981). Cyclobutane pyrimidine dimers (CPDs) have been shown to be induced both by UVC and UVB radiation (Pfeifer, 1997; Yoon *et al.*, 2000) but the latter at a yield of approximately 20 to 100-fold lower than UVC (Mitchell *et al.*, 1991; Perdiz *et al.*, 2000). However, UVA radiation (320-340nm) has been reported to give rise to T<>T cyclobutane dimers in Chinese hamster ovary cells most likely formed via a photosensitized triplet energy transfer (Douki *et al.*, 2003; Rochette *et al.*, 2003). It appears, therefore, that the polyclonal antiserum IgG479 could be applied in the assessment of DNA damage and repair in defined DNA sequences following exposure to either UVC or UVB radiation. This is very important particularly due to gene-specific induction of DNA damage by various genotoxins, such as ultraviolet radiation, and repair in several biological organisms (Bohr *et al.*, 1985; Mellon *et al.*, 1986). Another potential application of this antiserum might be as a sequence-specific probe in recognising damaged sequences flanked by one or more pyrimidines on either side in highly mutated genes such as the *p*53 tumor suppressor gene. Mutations in this gene are C→T transitions and CC→TT double transitions at dipyrimidine

sequences, which are specifically induced by UV-light (Nataraj *et al.*, 1995), and have been shown to occur in human skin cancers (Brash *et al.*, 1991). These damaged sequences could be identified using a sequence-specific antibody, such as the one described in this study. In addition, its potential use in the detection of repaired DNA lesions, such as T<>T, in the urine of psoriasis patients following treatment with UVB phototherapy will be investigated as further information regarding the effects of UVB-mediated genotoxicity might be obtained.

In conclusion, ELISA assay was found to be an effective method for the detection of UVR-induced DNA damage using a lesion-specific antibody. Low limits of detection of antisera to cyclobutane pyrimidine dimers were previously reported using ELISA. Mori *et al.* (1991) reported a detection limit of 0.28fmol of cyclobutane thymine dimers using a monoclonal antibody by ELISA. In addition, recent study by Cooke *et al.* (2003b) has shown the ELISA assay to have 10-25 times lower limits in the detection of cyclobutane thymine dimers using polyclonal antiserum 529 (0.9fmol of dimer) compared with GC-MS method (20-50 fmol dimer) previously described by Podmore *et al.* (1996).

CHAPTER 4: DETECTION OF UVR-INDUCED DNA REPAIR PRODUCTS BY POLYCLONAL ANTISERUM

CHAPTER 4

DETECTION OF UVR-INDUCED DNA REPAIR PRODUCTS BY POLYCLONAL ANTISERUM

4.1 INTRODUCTION

Strong epidemiological and clinical evidence have implicated exposure to ultraviolet radiation (UVR) as a major risk factor in the development of human skin cancer (Nataraj *et al.*, 1995). The nature of UVR-induced DNA damage depends on the wavelength range (Ravanat *et al.*, 2001). Direct light absorption by DNA results mainly in dimerisation reactions between adjacent pyrimidine dimers, giving rise to several types of lesions, including cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts ((6-4)PPs) formed by UVC (200-280nm) and UVB (280-320nm) radiation (Pfeifer, 1997). UVA radiation (320-400nm) is weakly absorbed by DNA but leads to DNA damage through excitation of endogenous chromophores as radiation absorbing intermediates, yielding oxidatively damaged products such as 8-oxo-2'-deoxyguanosine (8-oxodG) (Rosen *et al.*, 1996). However, UVA radiation has been reported to yield CPDs (Kvam and Tyrrell, 1997; Douki *et al.*, 2003), but much less efficiently compared to UVB radiation (Perdiz *et al.*, 2000). All these lesions are considered to be mutagenic (Tornaletti *et al.*, 1993) and therefore potentially carcinogenic (Brash *et al.*, 1991; Guyton and Kensler, 1993).

The efficient removal of DNA lesions is an important step in the prevention of tumour formation. Bulky helix distorting adducts are principally removed by the activities of nucleotide excision repair (NER) pathway, although faster repair of T<>T and 8-oxodG

has been reported to occur by transcription-coupled repair (TCR) (Van Hoffen *et al.*, 1995; Le Page *et al.*, 2000). Such repair processes are characterised by the removal of largely bulky adducts in the form of a single-stranded oligomer, about 24-29 bases long containing a lesion (Galloway *et al.*, 1994; Huang *et al.*, 1994). Such excised oligomers become subject to 5'→ 3' exonucleolytic attack that would continue until the lesion is encountered, resulting in a 6- or 7-mer and subsequently excreted in urine (Galloway *et al.*, 1994).

Detection of UVR-induced DNA lesions in urine is important in the study of psoriasis following phototherapy treatment, as further information about the mechanisms of UVR-induced mutagenesis or carcinogenesis and an individual's repair ability to process DNA lesions might be obtained. Psoriasis is a chronic inflammatory skin disease characterized by abnormal differentiation and hyperproliferation of human skin keratinocytes (Stern, 1997). Photochemotherapy, which combines psoralen with UVA radiation (PUVA), is one of the most effective treatments of psoriasis since its introduction in 1974 (Parrish *et al.*, 1974). Psoralens are a class of photo-mutagenic and photo-chemotherapeutic molecules that are found naturally and are used in conjunction with UVA to improve the efficiency of UVA phototherapy, an approach commonly known as PUVA (Gasparro, 1988). Psoralens become activated upon exposure to UVA light, after which they intercalate into DNA forming psoralen-DNA photoadducts (Stern *et al.*, 1979). Though important in the treatment of psoriasis, the safety of PUVA therapy has been an issue of concern and debate mainly due to its long-term side effects including an increased risk of cutaneous malignancy, such as squamous cell carcinoma (SCC) (Henseler *et al.*, 1987; Chuang *et al.*, 1992) and possibly melanoma (Stern *et al.*, 1997). This has lead to an increased use of UVB phototherapy. Narrow-band UVB (NB-UVB), such as that from Philips TL-01 lamps, which emit a narrow peak at 311 to 313 nm, is an effective treatment for moderate-

to-severe psoriasis and other skin conditions (British Photodermatology Group, 1997). NB-UVB permits rapid control of psoriasis and the risk of long term photocarcinogenesis may be reduced, due to the absence of UVB emitted in the erythematogeneous region of the UV spectrum, suggesting that more therapeutic UVB can be delivered before erythema occurs (Young, 1995). NB-UVB is considered to have significantly fewer side effects, including skin blistering, erythema and the arguably lower long-term cancer risk when compared to PUVA (Tanew *et al.*, 1999). Animal studies have determined that TL-01 lamps appear to be more effective than treatment with broadband UVB sources, such as TL-12 lamps. Furthermore, the median time for induction of tumours was significantly longer in animals exposed to TL-01 lamps, than in those exposed to the TL-12 lamps (Van Weelden *et al.*, 1988). Moreover, when compared with broad-band UVB, lower minimal erythema dose (MED)-equivalents of NB-UVB are required to clear psoriasis in humans, which may be associated with a reduced risk of carcinogenesis (Young, 1995).

Numerous studies have investigated the excretion of urinary lesions such as 8-oxodG and T<>T in urine. Gas chromatography/mass spectrometry (GC-MS) (Halliwell and Dizdaroglu, 1992; Ravanat *et al.*, 1999), high-performance liquid chromatography (HPLC) (Loft *et al.*, 1992; Tagesson *et al.*, 1992; 1995; Germadnik *et al.*, 1997; Bogdanov *et al.*, 1999) and HPLC with electrochemical detection (HPLC/EC) (Halliwell and Dizdaroglu, 1992; Evans *et al.*, 2000; Pilger *et al.*, 2001) have been widely used to measure urinary levels of 8-oxodG. In addition, ^{32}P-postlabelling (Devanaboyina and Gupta, 1996; Le Curieux and Hemminki, 2001) and alkaline elution techniques (Pflaum *et al.*, 1997) have been employed in the measurement of 8-oxodG and T<>T in urine. Though important in providing absolute identification and quantification of lesions, these methods implicate extensive sample clean-up procedures in order to eliminate analytical interference from the

complex urine matrix and are not readily amenable to routine clinical analysis. Immunochemical approaches to the detection and measurement of urinary DNA lesions offer a sensitive and specific approach to the study of DNA damage utilising lesion-specific antibodies. Urinary T<>T and 8-oxodG have been previously measured in the urine of psoriasis patients treated with psoralen and UVA by an enzyme-linked immunosorbent (ELISA) assay using specific antibodies to T<>T and 8-oxodG (Ahmad *et al.*, 1999; Cooke *et al.*, 2000a; 2001b). In this study, the use of such non-invasive assay in the measurement of 8-oxodG, an important biomarker of oxidative stress, and T<>T, a major photoproduct generated by UVR exposure, in the urine of psoriasis patients following NB-UVB phototherapy was investigated.

4.2 AIM

The aim of the work presented in this study was (i) the application of polyclonal antiserum IgG479 in the measurement of urinary UVB-induced DNA lesions by competitive ELISA (ii) to establish the relative effects of NB-UVB lamps compared to the ultraviolet A (UVA) component of psoralen plus UVA radiation (PUVA) treatment described by Cooke *et al.* (2001b), upon DNA damage and (iii) draw conclusions evidence relating to safety of therapy and mechanism of action.

4.3 METHODS

4.3.1 Narrow band UVB-irradiation of subjects

Patients (described in Section 2.1.6) were given a course of TL-01 therapy in accordance with the British Photodermatology Group Guidelines (Taylor *et al.*, 2002), using UVB-emitting fluorescent tubes (Philips TL-01) (Figure 4.1) following determination of the minimal erythema dose (MED). MED is the quantity of radiant energy required to produce the first perceptible, unambiguous redness reaction with clearly defined borders. Following a urine sample, the patients commenced the first dose of UVR therapy on day 1 receiving 70% of their MED, followed by twice-weekly exposures on days 1, 8, 10, 15, 17, 22, 24, 29, 31, 36 and 38. The doses given to patients with psoriasis were increased according to standard protocols, which were as follows: if the previous exposure had induced no detectable effect, the exposure time was increased by 50%, followed by a 30% increase in case of a slight erythema and the same exposure time in case of marked erythema.. The wavelength emission of the light source was validated using a photometer.

4.3.2 Measurement of 8-oxodG in urine

Measurements of levels of 8-oxodG in the urine of psoriasis patients was performed using 8-oxodG ELISA assay as previously described (Section 2.8.2-2.8.2.1). The resulting absorbances were read at 450nm using a plate reader (Anthos 2001). The amount of 8-oxodG present in the urine samples was determined using a standard curve. The standard curve was generated by plotting the absorbances obtained for 8-oxodG against log concentration of each of the 8-oxodG samples. Concentrations of the urine samples were then determined using the absorbance values obtained by ELISA.

4.3.3 Competitive ELISA for urinary T<>T

Competitive ELISA for the measurement of urinary T<>T in psoriatic patients was performed using a competitive ELISA in a manner similar to that described earlier (Section 2.7). Single-stranded, UVC-DNA (0.882KJ/m^2) was used as the solid phase antigen and bound to a 96-well poly-L-lysine coated ELISA plate at 50μg/ml in PBS (50μl/well). All incubations were carried out at 37°C for one hour in a humidified chamber. Following washing of the wells with PBS and blocking of free sites with milk powder solution (200μl/well), 50μl/well of urine samples were then applied to the plate, along with 50μl/well of the diluted IgG479 (1:5000 in 4% w/v dried milk/PBS). Detection of bound antiserum was achieved using peroxidase-labelled goat anti-rabbit immunoglobulin (IgG) diluted 1:2000 (in 4% w/v dried milk/PBS; 100μl/well) in conjunction with orthophenylenediamine (0.5mg/ml in 0.05M phosphate-citrate, pH 5.0, and containing 0.03% w/v sodium perborate; 50μl/well) substrate solution. The reaction was stopped using 2M H_2SO_4 (100μl/well) and the resulting absorbance was read at 492nm using a plate reader (Anthos 2001).The results were expressed as percentage inhibition using the formula described previously in Section 2.7.

4.3.4 Urinary Creatinine Measurement

In order to correct for variations in urine concentration, the urinary creatinine was assessed by a procedure based upon the Jaffe reaction (Henry, 1974) where 2mls of each of the urine samples were analysed by the Department of Chemical Pathology, Leicester Royal Infirmary NHS Trust, Leicester, U.K. The results obtained for urinary 8-oxodG and T<>T by ELISA were expressed per unit creatinine.

4.3.5 Statistical Analysis

Statistical analysis for urinary 8-oxodG and T<>T levels was performed using *t* test between individuals, GraphPad Software Prism, version 2.01 (GraphPad Software, San Diego, CA), and Microsoft Excel 2000.

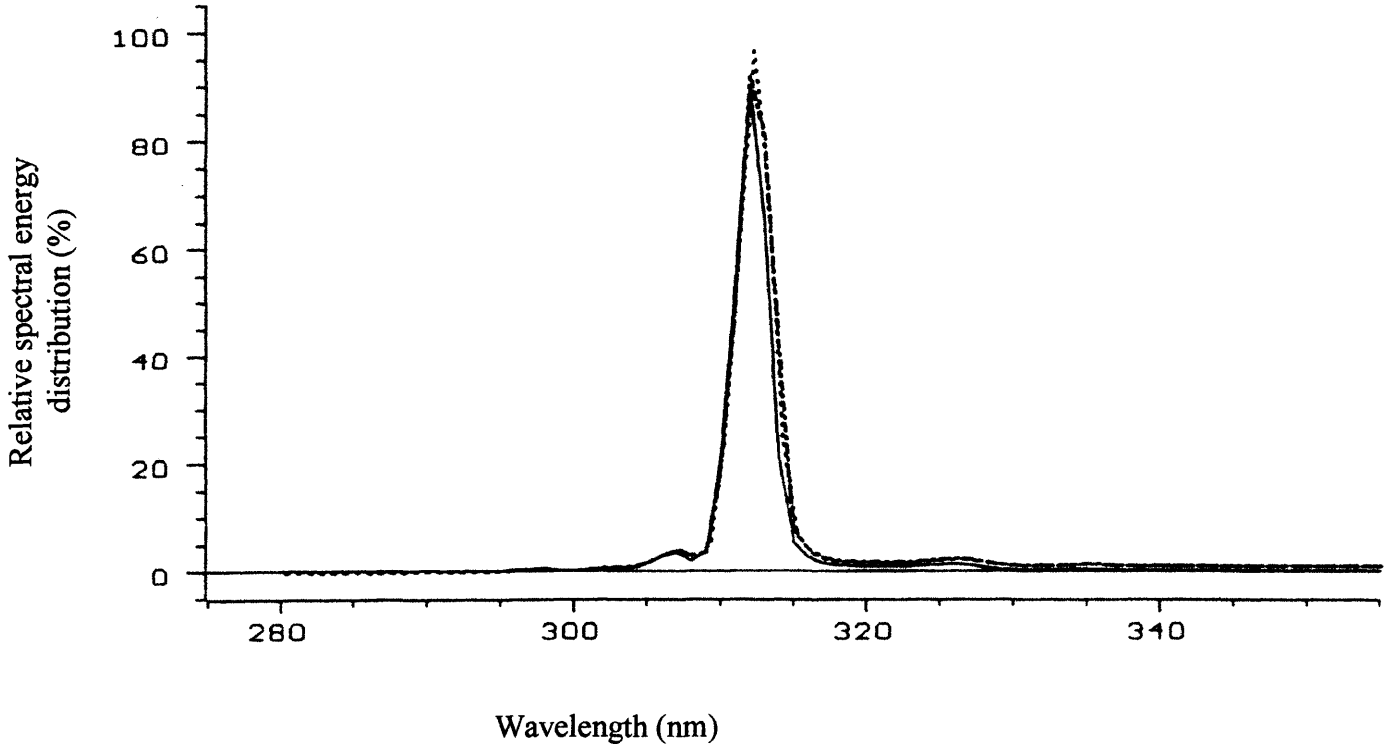


Figure 4.1: Spectral characteristic of UVB Philips TL-01 lamp. Irradiant intensity is plotted against wavelength. The continuous curve shows UVB-emission wavelength at 311nm whereas the dotted curve shows wavelength at 313nm (adapted from Verre & Quartz Dixwell, France).

4.4 RESULTS

In this study, measurements of 8-oxodG and T<>T in the urine of patients with guttate or plaque psoriasis following narrow-band UVB treatment was performed by competitive ELISA using polyclonal antiserum IgG479. The competitive ELISA for T<>T was performed according to the method described in Ahmad *et al.* (1999), where validation results in terms of inter- and intra-plate variability demonstrated the assay to be suitable for measurement of clinical samples and the simultaneous study of a large number of samples in its current format (Cooke *et al.*, 2001b). The intra-assay coefficient of variation was 5.63%, whereas inter-assay variability was shown to be 10.30% (Cooke *et al.*, 2001b). Results obtained from both ELISA assays, one for 8-oxodG and the other for T<>T, were expressed as percentage inhibition, corrected for creatinine in order to allow direct comparison between the urinary DNA lesions. This was due to the absence of a calibration graph for the T<>T assay compared to that for the 8-oxodG, where 8-oxodG standards were used in the calibration of the assay. The 8-oxodG ELISA is a well established assay with inter- and intra-assay variability of less than 10% for 8-oxodG, as determined by the manufacturer (JaICA). In addition, Cooke *et al.* (2000a) reported great consistency of baseline levels for 8-oxodG in the urine of healthy individuals measured by ELISA-based assay.

Urinary 8-oxodG and T<>T have been previously measured in psoriatic patients (Ahmad *et al.*, 1999), as well as individuals exposed to a UVA source used in PUVA therapy (Ahmad *et al.*, 1999; Cooke *et al.*, 2001b), using an ELISA assay, suggesting that measurement of these lesions in urine could be used to biomonitor UVR-mediated genotoxic insult. In this study, measurement of the efficiency of NB-UVB lamps compared to PUVA treatment and the different risk/benefits of each therapy was investigated.

Though a small number (thirteen) of samples was examined in this study, highly significant results were obtained by statistical analysis for the urinary T<>T lesions. Previous study on the measurement of these DNA lesions in the urine of healthy, human volunteers following exposure to a UVA source used in PUVA therapy was performed using just fourteen subjects (Cooke *et al.*, 2001), resulting in highly significant results using summary statistical analysis.

Assessment of urinary creatinine concentration indicated the patients with psoriasis to be free from renal disease, enabling meaningful analysis of urinary T<>T and 8-oxodG (Figure 4.2). Baseline levels of both T<>T and 8-oxodG, in control, unirradiated individuals were consistent with those reported by Ahmad *et al.* (1999), (6.46 +/- 2.99 percentage inhibition/creatinine, and 4.48 +/- 2.45 pmol 8-oxodG/μmol creatinine, respectively). The absence of significant variation in urinary 8-oxodG output in control, unirradiated individuals over a similar, albeit shorter, period of study has been previously described (Cooke *et al.*, 2001). Nevertheless, appreciable inter-and intra-time point variability was demonstrated (Figure 4.3), consistent with the findings of Pilger *et al.* (2001), although no significant differences in urinary 8-oxodG levels were noted over the 45 day period of study. In contrast, statistical analysis of urinary T<>T levels revealed a significant ($p = 0.05$) increase at day 7 following irradiation (Figure 4.4), which was followed by a further significant increase ($p = 0.05$) at day 14. Maximal excretion, over the period studied, appeared to be at day 45 ($p<0.0001$). No significant differences in levels of urinary 8-oxodG were seen over the period of study.

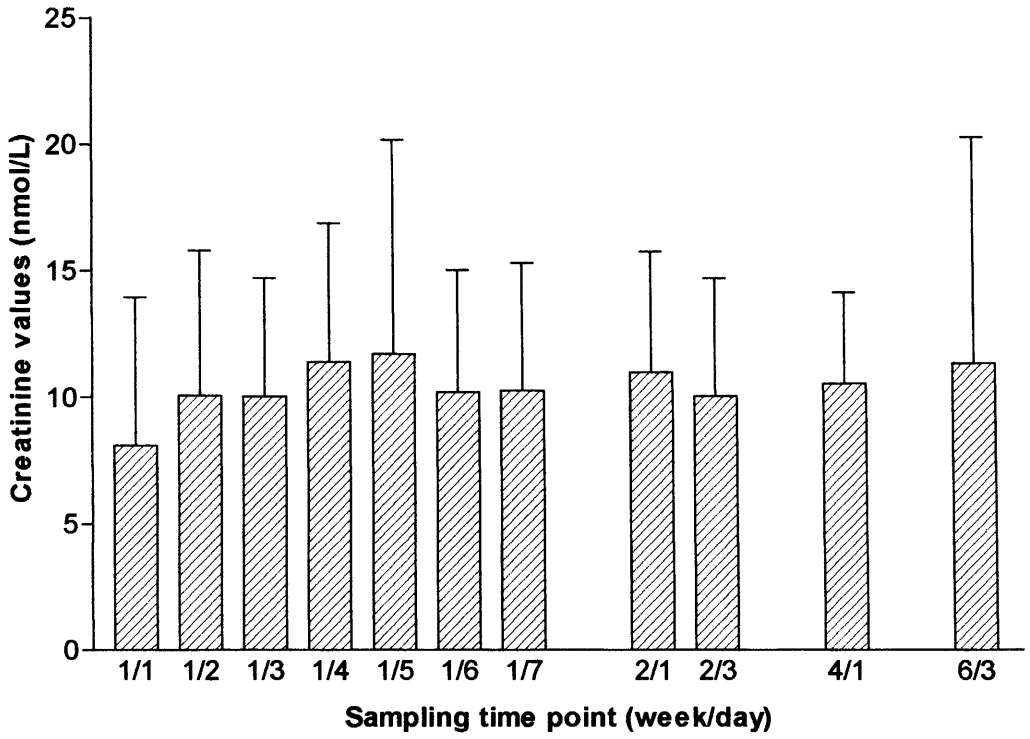


Figure 4.2: Creatinine analysis of urinary 8-oxodG and T<>T. Urinary creatinine values (nmol/L) of all patients with guttate or plaque psoriasis were examined and plotted against corresponding sampling time point (week/day) over 45 day period of study. Bars represent the mean (± standard deviations) for 3 samples. Statistical analysis by *t* test failed to demonstrate any significant day-to-day differences.

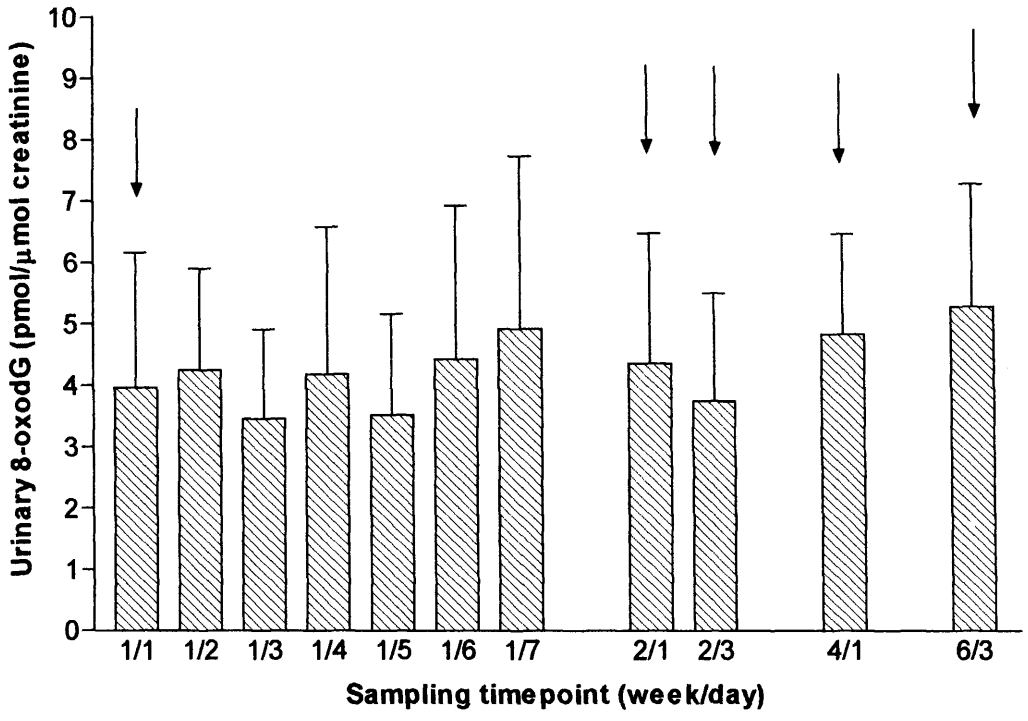


Figure 4.3: Competitive ELISA analysis of urinary 8-oxodG. Thirteen human volunteers with guttate or plaque psoriasis were exposed to narrow-band UVB lamps, according to the regime described in the main text. Daily urine samples were collected post UVB exposure over a period of 45 days. Values are corrected for creatinine (pmol/µmol) and expressed as urinary 8-oxodG inhibition. Bars represent the mean (± standard deviations) for 3 samples. Arrows indicate the days of exposure of psoriatic patients to narrow-band UVB lamps. Statistical analysis was performed using *t* test.

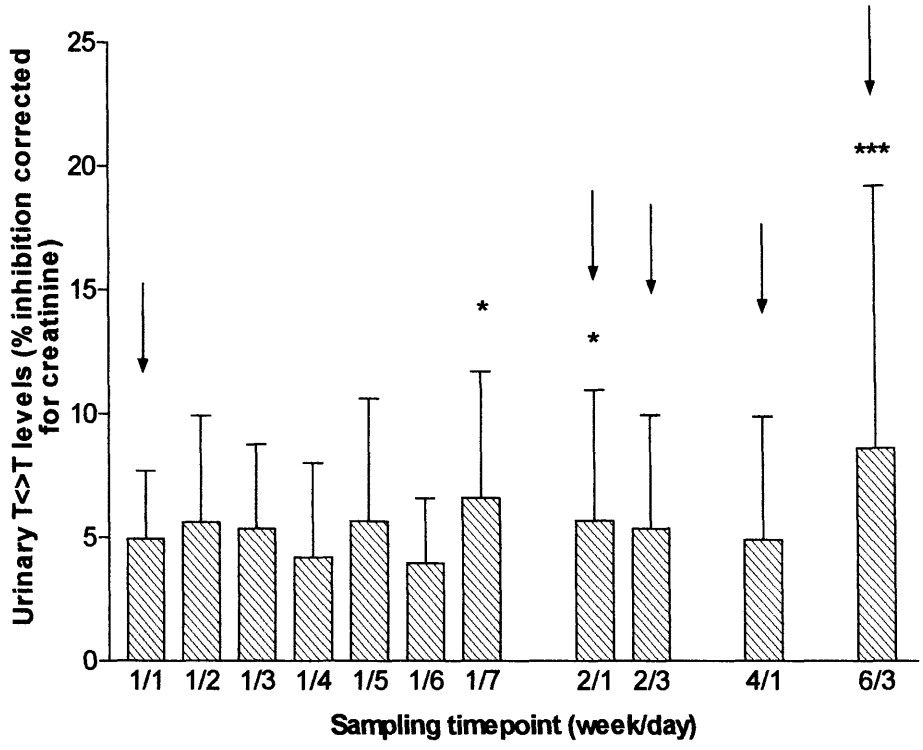


Figure 4.4: Competitive ELISA of urinary T<>T. Thirteen human volunteers with guttate or plaque psoriasis were exposed to narrow-band UVB lamps. Daily urine samples were collected post UV exposure over a period of 45 days. Values are corrected for creatinine (pmol/μmol) and expressed as urinary T<>T inhibition. Bars represent the mean (± standard deviations) for 3 samples. Asterisks indicate a statistically significant (*p = 0.05 and ***p<0.0001) difference compared to baseline, determined by *t* test. Arrows indicate the days of exposure of psoriatic patients to narrow-band UVB lamps.

4.5 DISCUSSION

PUVA is a well-established treatment for psoriasis, but is associated with an increased risk of carcinogenesis, related, in part, to the cumulative dose of UVA received during PUVA therapy (Chuang *et al.*, 1992; Stern *et al.*, 1997). NB-UVB phototherapy is a standard treatment for chronic plaque and guttate psoriasis associated with a lower risk of developing skin cancer (Van Weelden *et al.*, 1988). In this study urinary 8-oxodG and T<>T were measured in patients with guttate or plaque psoriasis following narrow-band UVB exposure over a period of 45 days. No changes in urinary creatinine levels were noted during the period of study. Creatinine is a well-established marker used routinely to correct for variations in urine concentration and it is considered to act as an indicator of renal function (Cooke *et al.*, 2000a; 2001b). In addition, 8-oxodG/creatinine levels and a small number of spot urine samples corrected for creatinine were shown to be rather stable in an individual over time (24 h) indicating low intra-variability as described by Bogdanov *et al.* (1999).

A competitive ELISA was used in the detection of T<>T in the urine of psoriasis patients utilising polyclonal antiserum IgG479, which was shown to be specific for T<>T (Ahmad *et al.*, 1999). The results demonstrated a peak of excretion of T<>T in urine samples 7 days after initial exposure to TL-01 lamps followed by a second increase at day 14. However, a maximal increase in levels of urinary T<>T was detected at day 45, associated with the increasing dose of UVB used in the treatment protocol.

Increases in urinary levels of T<>T were noted for a number of days post-irradiation, consistent with other studies, postulated to be due to a delay between lesion induction, repair, processing and appearance in the urine (Cooke *et al.*, 2001b). Conversely, no increases in urinary 8-oxodG were noted at any time post-irradiation, in contrast to

previous findings with a UVA source (Cooke *et al.*, 2001b). From this it may be concluded that the emission peak at 311 nm of the TL-01 lamp, whilst effective at inducing T<>T, precludes the formation of oxidative DNA lesions, such as 8-oxodG. Moreover, the two increases in the urinary T<>T levels detected at days 14 and 45 could be random and the result of wide variability observed in data (Figure 4.4).

A number of reports confirm the production of significant levels of oxidative DNA damage in cells, following UVA exposure (Kvam and Tyrrell, 1997). Whilst the quantum yield of 8-oxodG at 311nm may be less than that of pyrimidine dimers (Enninga *et al.*, 1986; Kvam and Tyrrell, 1997) there are, nonetheless, descriptions of broad-band UVB sources (280-320 nm) which induce appreciable amounts of 8-oxodG in epidermal cells of hairless mice, after chronic exposure (Hattori *et al.*, 1997). However, Hattori *et al.* suggest that at least a proportion of this is derived from erythema-associated inflammation (1997). Given that DNA weakly absorbs UVA radiation (320-400nm) (Sutherland and Griffin, 1981), T<>T production by UVA radiation is likely to be caused *via* a photosensitized-based mechanism. Indeed, recent data by Douki *et al.* showed the formation of T<>T cyclobutane dimers in Chinese hamster ovary cells following irradiation with pure UVA radiation (340-400nm) *via* a photosensitized triplet energy transfer from an excited chromophore to thymine (2003). Since narrow band UVB minimises erythema, this may, in part, account for the absence of 8-oxodG induction with this form of therapy. There is growing evidence for the important role of oxidative DNA damage in mutagenesis and carcinogenesis (Cooke *et al.*, 2003a). The involvement of elevated levels of such damage, in conjunction with dimeric and PUVA-related lesions, may account for the increased risk of carcinogenesis associated with PUVA therapy.

In summary, the induction and repair of DNA lesions in the urine of psoriatic patients following treatment with narrow band UVB phototherapy was established by competitive ELISA utilizing polyclonal antiserum IgG479. Results obtained from competitive ELISA showed the induction and repair of T<>T cyclobutane dimers, in the absence of 8-oxodG, by NB-UVB lamps, and their excretion in urine of psoriatic patients. This observation suggested that the therapeutic TL-01 sources used in NB-UVB phototherapy may deliver their effectiveness from the induction of T<>T, and that the relatively low cancer risk, compared to the therapeutic source used to provide UVA for PUVA treatment, may derive from the lack of 8-oxodG induction. The employment of a polyclonal antiserum in the measurement of UVB-induced T<>T as repaired products in the urine of patients with psoriasis support the application of urinary DNA damage assessments to the non-invasive biomonitoring of phototherapies, which may result in the improved evaluation of risk/benefit to the patient. Finally, these data provide insight into the potential mechanism through which phototherapies exert their beneficial and side-effects.

CHAPTER 5: DEVELOPMENT OF AN IMMUNOASSAY FOR THE DETECTION AND QUANTIFICATION OF UVC-INDUCED DNA DAMAGE IN GENE-SPECIFIC SEQUENCES

CHAPTER 5

DEVELOPMENT OF AN IMMUNOASSAY FOR THE DETECTION AND QUANTIFICATION OF UVC-INDUCED DNA DAMAGE IN GENE-SPECIFIC SEQUENCES.

5.1 INTRODUCTION

Most of the work in DNA damage and repair over the last decades has been carried out by measuring the events in the total genome. However, studies on carcinogens, such as ultraviolet radiation (UVR), have shown that induction of DNA damage and repair does not occur equally throughout the entire genome (Bohr *et al.*, 1985), but varies according to factors such as DNA sequence, chromatin structure, characteristics of the proteins involved in the repair of DNA damage and transcriptional activity of actively expressed genes (Mellon *et al.*, 1987; Bohr *et al.*, 1987; Sendowski and Rajewsky, 1991). UVR-induced pyrimidine dimers, for example, are repaired more efficiently in actively transcribed genes compared to non-transcribed regions of the genome (Bohr *et al.*, 1985; Mellon *et al.*, 1987). This gene-specific repair was shown to be due to the preferential removal of pyrimidine dimers from the transcribed strand (Mellon *et al.*, 1987). Preferential repair of transcribed genes, in particular, has been shown to correlate with the sensitivity of cells to various DNA damaging agents, such as UVR radiation (Bohr *et al.*, 1985; 1987). As a consequence, analysis of these parameters at a global genome level may not reflect important gene-level events.

Few techniques have been established to explore quantitatively gene-specific DNA damage and repair. Most of these are polymerase chain (PCR)-based assays (Govan *et al.*, 1990; Jennerwein and Eastman, 1991; Kalinowski *et al.*, 1992), which although very sensitive,

require high levels of lesions in the target DNA region in order to obtain a quantifiable decrease in amplification. The ligation-mediated polymerase chain reaction (LM-PCR) is a method for detecting and mapping the frequency of DNA damage products and has been used to map UVR-induced DNA damage (Tornaletti *et al.*, 1993; Tornaletti and Pfeifer, 1994; Gao *et al.*, 1994) and bulky mutagen-induced DNA damage (Denissenko *et al.*, 1996b) at single nucleotide resolution. However, this technique is limited by the requirement for high doses of mutagens, which are often cytotoxic to mammalian cells. In addition, the assay appears most suited for mapping lesions and not quantification.

Antibodies raised against DNA lesions have been applied to the detection and quantification of DNA damage within target gene sequences using sequence-specific ALISS (Adduct Levels in Specific Sequences) assay (Hochleitner *et al.*, 1991; Thomale *et al.*, 1994) and immuno-coupled PCR (ICPCR) (Denissenko *et al.*, 1994; 1996).

In this study, the potential use of ICPCR assay in the detection of UVC-induced DNA damage in gene-specific sequences within the human H-*ras* proto-oncogene was investigated. Activating mutations of the H-*ras* gene were shown to occur in non-melanoma skin cancer in a frequency of 30-50% (Barbacid, 1990), suggesting H-*ras* gene as a suitable model in UV-radiation studies.

5.2 AIM

The aim of the work presented in this chapter was (i) the development of an immunoassay (ICPCR) to detect and quantify the induction of T<>T in the human H-*ras* gene following UVC radiation and (ii) the application of ICPCR to compare gene and global measures of T<>T within human genomic DNA.

5.3 METHODS

5.3.1 UVC-irradiation of DNA

A solution of human genomic DNA at a concentration of 1mg/ml in TE buffer (10mM Tris-HCl, pH 7.5 and 1mM EDTA) and calf thymus DNA [0.5mg/ml in phosphate-buffered saline (0.01M, pH 7.4)] were exposed to increasing doses of UVC radiation (0-3.48KJ/m^2) in a plastic tissue culture plate (previously described in Section 2.5). Irradiations were performed at a distance of 6 cm from the UVC lamp and on ice to prevent heating effects as previously described (Section 2.3.1).

5.3.2 DEVELOPMENT OF IMMUNO-COUPLED POLYMERASE CHAIN REACTION

5.3.2.1 Optimisation of PCR conditions and primer specificity

PCR amplification of human genomic DNA [UVC-irradiated (1.47KJ/m^2) and unirradiated, *Bam*HI-digested DNA; 50ng per reaction] was performed using HotStartTaq DNA polymerase and primers H-*ras*7 and H-*ras*8 as described in the general protocol (Section 2.11). A negative control consisting of nuclease-free PCR grade water and all the PCR reagents but no DNA was included in the PCR. Following amplification, the PCR products were analysed on a 2.0% agarose gel along with the 123bp DNA Ladder (Section 2.12).

5.3.2.2 PCR amplification of human genomic DNA

Human genomic DNA samples (UVC-irradiated, *Bam*HI digested genomic DNA) were PCR amplified using HotStartTaq DNA polymerase as previously described (Section

2.11). PCR positive (unirradiated, *Bam*HI digested DNA samples) and negative controls (no DNA just PCR reagents) were PCR amplified in the same way and included in each PCR. Following amplification, PCR products were analysed as described earlier (Section 2.12). Human genomic DNA samples (unirradiated, *Bam*HI-digested; 0-5ng), which were PCR amplified alongside the test DNA samples and run in parallel, were used as standards in the quantification of PCR products by comparing the band intensity of the 149bp PCR product. Statistical analyses were performed using GraphPad Prism, version 2.01 (GraphPad Software, San Diego, CA).

5.3.2.3 Investigation of thermal cycling conditions using real-time PCR

Real-Time PCR was performed using Brilliant® SYBR® Green Quantitative PCR Core Reagent Kit (Stratagene) as described in Section 2.13. UVC-irradiated (1.47KJ/m^2), *Bam*HI-digested human genomic DNA (50ng per PCR) was amplified in the MX4000® Multiplex Quantitative PCR System (Stratagene).

5.3.3 Investigation of the efficiency of protein A and Blotto blocker by (i) ELISA and (ii) ICPCR

5.3.3.1 (i) ELISA assay

ELISA plates were coated either with protein A (100µg/ml in PBS; 100µl/well) or without it [PBS was added instead (0.01M; pH 7.4; 100µl/well)] and incubated at 4°C overnight in a humidified chamber. Calf thymus DNA [UVC-irradiated and native (non-irradiated) DNA; 0.5mg/ml in PBS] solutions were made single-stranded by heat denaturing for 10 minutes in a screw cap Eppendorf tube in a water bath followed by a rapid cooling on ice. Single-stranded, UVC-irradiated (0.882KJ/cm^2) calf thymus DNA was used as the solid phase antigen and bound to a 96-well ELISA plate at 50µg/ml in PBS (50µl/well). All

incubations were carried out at 37°C for one hour in a humidified chamber. Following three washes in PBS, free sites were blocked by incubation either with Blotto blocker (20mM Tris-HCL, pH 8.0; 120mM glycine; 5% dry milk powder; 0.5% gelatin; 0.01% thimerosal; 0.01% antifoam-A and 0.1% Tween 20) solution (200µl/well) or without it (PBS was used instead; 200µl/well). Polyclonal antiserum (IgG479) was diluted 1:2000 in 4% w/v milk/PBS and 50µl/well was added to the plate washed with PBS. Peroxidase-labelled goat anti-rabbit immunoglobulin (IgG) was used at a dilution of 1:2000 in milk/PBS (50µl/well), following washing of wells three times with PBS containing 0.05% (v/v) Tween 20. Detection of bound peroxidase-labelled antibody was performed using 50µl/well of orthophenylenediamine (0.5mg/ml in 0.05M phosphate-citrate, pH 5.0 and containing 0.03% w/v sodium perborate) as substrate solution. The reaction was stopped after 15-minute incubation at room temperature using 2M H_2SO_4 (25µl/well). Absorbances were read at 492 nm using a plate reader (Anthos 2001, Anthos Labtec Instruments) and analysis was performed by GraphPad Software Prism, version 2.01.

5.3.3.2 Determination of the working volume of blocking and washing solutions

Different volumes of Blotto blocker (50-500µl per tube), PBS and Tris-HCl (10mM; pH 8.3) (100-500µl per tube) were tested by ICPCR using UVC-irradiated (1.47 KJ/m^2) and unirradiated, *Bam*HI-digested human genomic DNA. ICPCR was performed in a manner similar to that described in the general protocol (Section 2.14) and PCR products were analysed on a 2.0% agarose gel followed by staining of the gel with ethidium bromide (5.0µg/ml in distilled water) and visualisation by ultraviolet light.

5.3.3.3 (ii) ICPCR assay

Immuno-coupled PCR assay was performed according to the general protocol but the following modifications were included in order to investigate the importance of protein A in antiserum binding and the efficiency of Blotto blocker in blocking any non-specific binding sites available in the tubes. Tubes (0.5ml Gene Amp® Reaction tubes) were coated either with or without protein A (100μg/ml of protein A in sodium bicarbonate buffer, pH 9.5; 100μl per tube) and blocked either with or without Blotto blocker solution (500μl Blotto blocker per tube; 20mM Tris-HCL, pH 8.0; 120mM glycine; 5% dry milk powder; 0.5% gelatin; 0.01% thimerosal; 0.01% antifoam-A and 0.1% Tween 20). All incubations were performed according to the general protocol described earlier (Section 2.14). Control unirradiated or UVC-irradiated (1.47KJ/m^2), *Bam*HI-digested human genomic DNA in PBS containing 0.5M NaCl was added to the tubes and incubated overnight at 4°C. Human genomic DNA samples were PCR amplified using HotStartTaq DNA polymerase as previously described (Section 2.11) and the PCR products were analysed on a 2.0% agarose gel along with the 123bp DNA Ladder (Section 2.12). Quantification of the PCR bands was achieved by scanning the gels with a laser scanner and integrating the data for peak areas with computer assisted software (QuantiScan BIOSOFT^R software, version 2.1) (Section 2.12).

5.3.4 Investigation of the specificity of polyclonal antiserum to recognise UVC-induced DNA damage in the human H-*ras* gene by ICPCR assay

Following overnight incubation at 4°C, protein A coated tubes (100μg/ml of protein A in sodium bicarbonate buffer, pH 9.5; 100μl per tube) were incubated with antiserum IgG479 (1mg/ml in PBS; 100μl per tube) for 4 hours at 4°C. Following washing with PBS and

blocking with Blotto blocker, unirradiated and UVC-irradiated (1.47KJ/m^2), *Bam*HI-digested genomic DNA in PBS containing 0.5M NaCl was added to the tubes (50ng). An extra control tube including UVC-irradiated, *Bam*HI-digested genomic DNA without antiserum was also included and PCR amplified in a similar manner as the above samples. PCR amplification of genomic DNA samples was performed using HotStartTaq DNA Polymerase in the same way described earlier (Section 2.11). PCR digests were analysed on a 2.0% agarose gel and quantified with QuantiScan BIOSOFT^R software (Section 2.12). Statistical analyses were performed using GraphPad Prism, version 2.01.

5.3.5 Immuno-coupled PCR assay

ICPCR assay for human genomic DNA irradiated with increasing doses of UVC radiation (0-3.48KJ/m^2) was performed as previously described in the general protocol (Section 2.14). Varying amounts of UVC-irradiated (1.47KJ/m^2), *Bam*HI-digested human genomic DNA (0-100ng) and different doses of UVC-radiation (0-1.470KJ/m^2) were used in the ICPCR assay in order to establish the sensitivity of the assay. The irradiations being performed as described in Section 2.5.

5.3.6 Measurement of UVC-induced damage in the global genome by competitive ELISA

DNA damage induced in the global genome was measured by competitive ELISA in a manner similar to that described in Section 2.7. Single-stranded, UVC-irradiated (0.882KJ/m^2) calf thymus DNA was used as the solid phase antigen and serially diluted solutions of UVC-irradiated (0-3.48KJ/m^2) human genomic DNA (25µl/well) were used as competitors along with the IgG479 antibody (25µl/well). The resulting absorbance was read at 492nm using a plate reader (Anthos 2001) and the results were expressed as

percentage inhibition of antibody binding to the solid phase antigen by competitive inhibition using the formula previously described (Section 2.7). Statistical analysis was performed by GraphPad Software Prism, version 2.01, and Microsoft Excel 2000.

5.3.7 Quantification of T<>T in the global genome by competitive ELISA

Competitive ELISA was performed in a manner similar to that described above using an established concentration of human genomic DNA in the measurement of UVC-induced T<>T in the global genome. Briefly, single-stranded, UVC-irradiated, calf thymus DNA was used as the solid phase antigen and bound to an ELISA plate. Solutions of UVC-irradiated (0-3.48KJ/m^2), human genomic DNA (0.195μg/ml in 4% w/v dried skimmed milk/PBS) were applied to the plate (25μl/well), along with the IgG479 (1:5000 in 4% w/v dried milk/PBS; 25μl/well). Detection of primary antiserum was achieved as previously described (Section 2.7) and the absorbances were read at 492nm using Anthos 2001 plate reader. The results were expressed as percentage inhibition and analysed by GraphPad Software Prism, version 2.01, and Microsoft Excel 2000.

5.4 RESULTS

5.4.1 Establishment of PCR conditions and primer specificity

The thermal cycling conditions, PCR reaction buffer, primer design and concentration, amount and quality of template DNA are all essential factors in the establishment of a successful amplification. Human genomic DNA (type XIII, from human placenta) was certified to be free from RNase and DNase inhibitors (manufacturer's certificate) and its purity was measured using UV/spectrophotometry, which revealed an absorbance ratio at 260/280nm of 1.8, denoting pure DNA. PCR assays depend critically on accurate DNA concentration, thus care was taken to ensure precise measurements in each experiment.

PCR amplification of UVC-irradiated and unirradiated, *Bam*HI-digested human genomic DNA samples was performed using HotStartTaq DNA polymerase and gene-specific primers (H-*ras* 7 and 8). This set of primers was reported to result in amplification of the 149bp product (1621 to 1770) of exon 1 of the human H-*ras* gene (Denissenko *et al.*, 1994) coding for amino acids 1-34 (Figure 5.1).

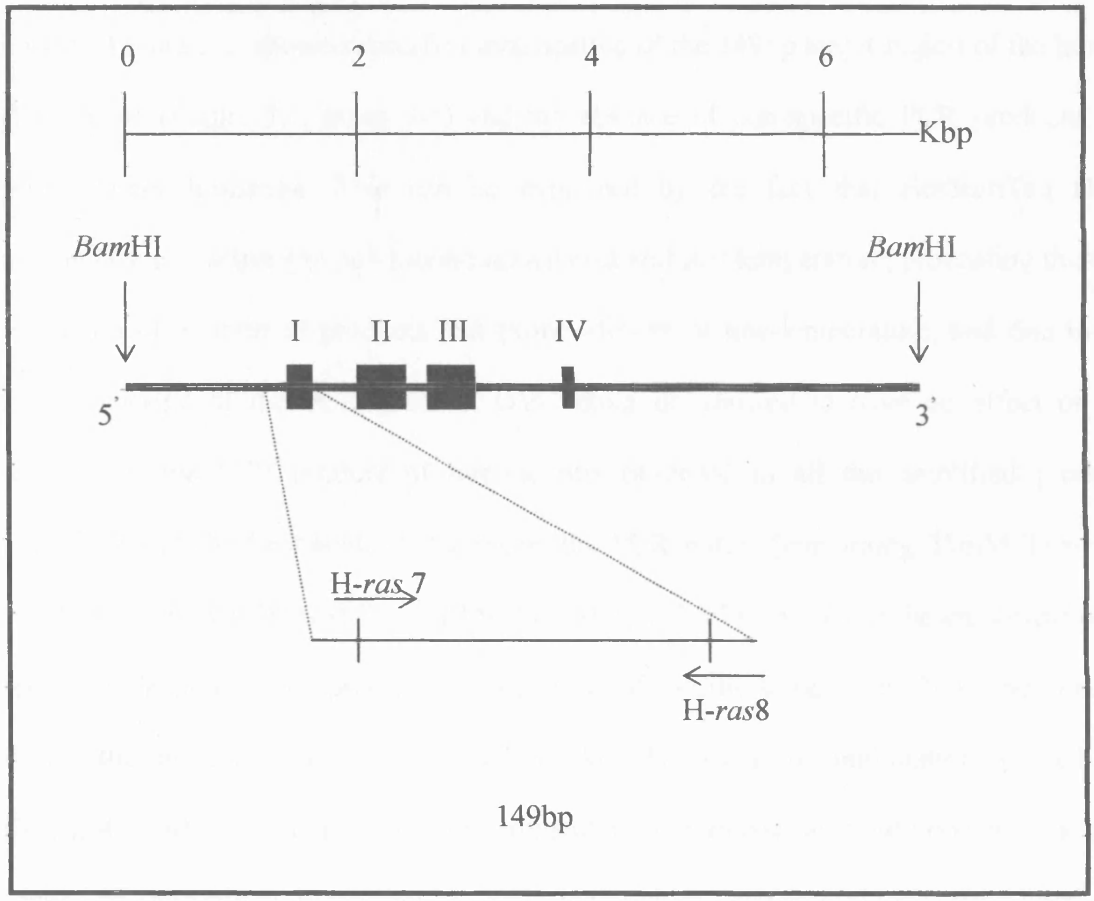


Figure 5.1: Map of human H-*ras* proto-oncogene. The exons are shown by Roman numbered boxes and the primer locations are indicated by horizontal arrows. The 149bp PCR amplified product includes codon 12 of exon 1. (Denissenko *et al.*, 1994)

Analysis of PCR products by agarose gel electrophoresis along with the 123bp DNA Ladder (Figure 5.2) showed specific amplification of the 149bp target region of the human H-*ras* gene (Figure 5.3; lanes 3-5) and the absence of non-specific PCR products and primer-dimer formation. This can be explained by the fact that HotStartTaq DNA polymerase is inactive (no polymerase activity) at ambient temperature, preventing thus the formation of misprimed products and primer-dimers at low-temperature, and due to the specific design of the PCR primers. UVC radiation showed to have no effect on the reaction as the PCR product of interest was observed in all the amplified products irrespective of the treatment. Furthermore, the PCR buffer [containing 25mM Tris-HCl, 50mM KCl, 50mM $(NH_4)_2SO_4$, and 15mM $MgCl_2$; pH 8.7] resulted in the amplification of specific PCR products by promoting a high ratio of specific-to-non-specific primer binding during the annealing step in each PCR cycle. The balanced combination of KCl and $(NH_4)_2SO_4$ buffers, which results in stringent primer-annealing conditions over a wide range of annealing temperatures and magnesium concentrations, eliminated the optimisation of PCR by varying the annealing temperature or the magnesium concentration. PCR amplification of human genomic DNA was thus, performed using 1.5mM magnesium concentration and an annealing temperature of 55°C. No band was detected for the negative PCR controls (lanes 1-2), confirming the specificity of the PCR conditions and their use in all subsequent experiments.

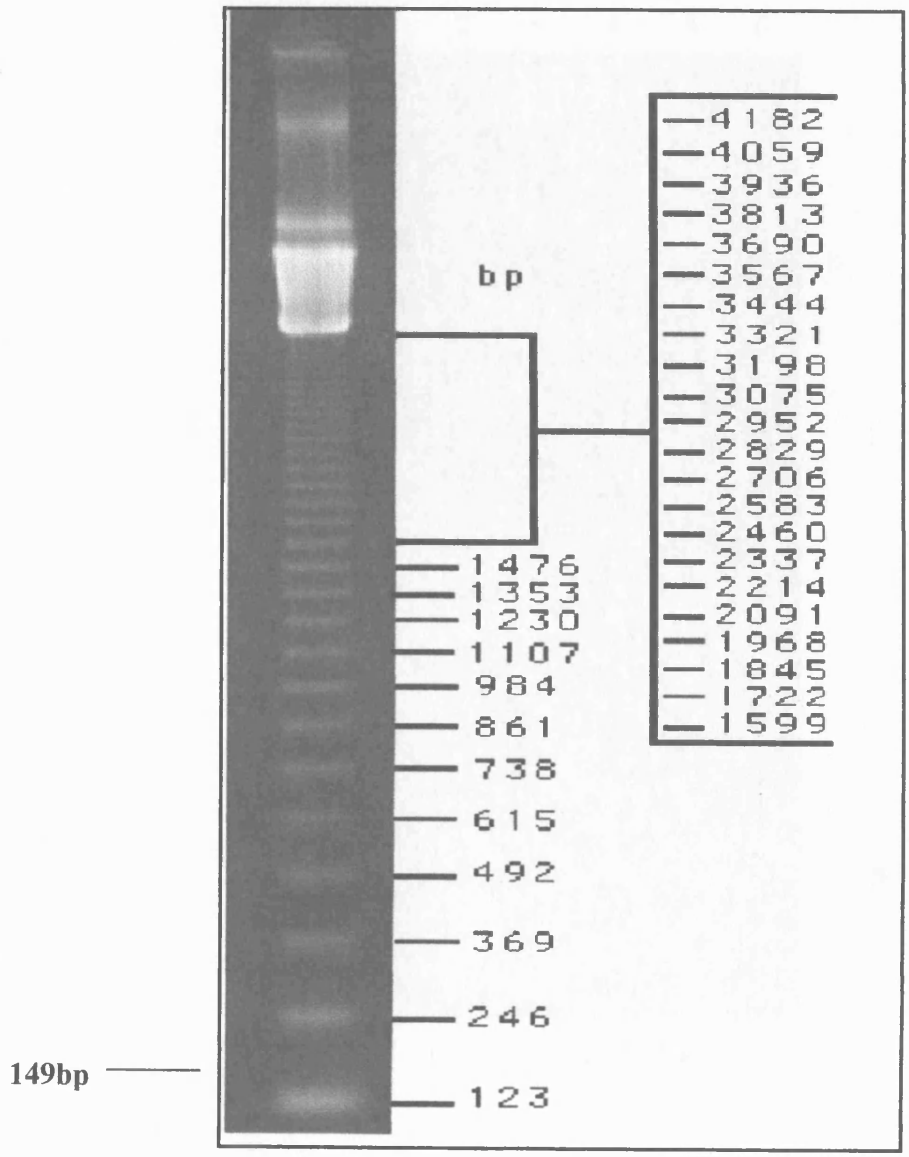


Figure 5.2: 123bp DNA Ladder. The figure shows a series of fragments ranging in length from 123 to 4182bp. Up to 25 separate bands could be seen on a 2% agarose gel after ethidium bromide staining. The 149bp band of interest appears just after the 123bp band.

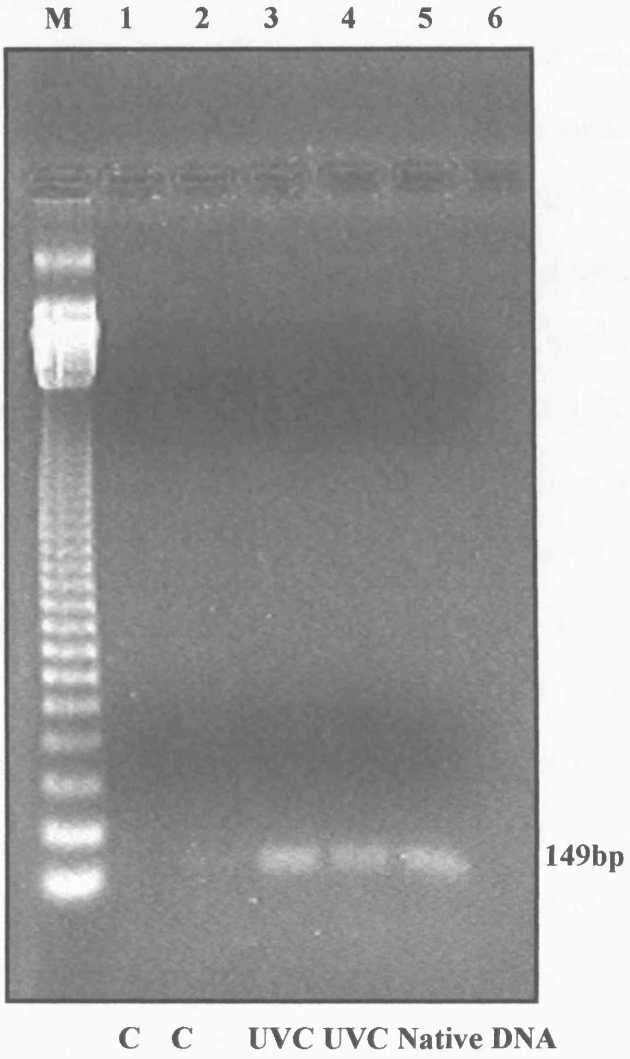


Figure 5.3: Representative gel showing PCR amplification of human genomic DNA using HotStartTaq DNA polymerase. Lanes 1-2 show negative PCR control (no DNA samples). Lanes 3-4 show 50ng of UVC-irradiated (1.47KJ/cm^2 of UVC-irradiation), *Bam*HI-digested DNA. Lane 5 shows 50ng of native (unirradiated) *Bam*HI-digested DNA. 40µl PCR aliquots were used. M, 123bp DNA Ladder.

5.4.2 Thermal cycling conditions

The thermal cycling profile (30 cycles) employed for the PCR amplification of gene-specific sequences within the human H-*ras* proto-oncogene was performed as previously described by Denissenko *et al.* (1994) and its efficiency was determined using real-time QPCR (Figure 5.4). UVC-irradiated, *Bam*HI-digested human genomic DNA (50ng per PCR reaction) was amplified using SureStart^{R}Taq DNA polymerase in the MX4000$^{®}$ Multiplex Quantitative PCR System (Stratagene). Real-Time Quantitative PCR was employed for detection of PCR amplification during early phases of the reaction. As it is shown on Figure 5.5, amplification of human genomic DNA occurred exponentially, that is a doubling of product (amplicon) occurs in every cycle (assumed 100% reaction efficiency). During this period there is still excess of all the PCR reagents and the enzyme is fully active. However, as the reaction progresses, some of the reagents are being consumed as a result of amplification leading to a slow down of the reaction and the PCR product is no longer being doubled at each cycle (linear phase of the reaction) until it reaches a plateau phase where there is little or no net increase in PCR product due to degradation of reactants (dNTPs, enzyme), depletion of primers, end-product inhibition and incomplete denaturation at higher temperatures (Figure 5.5). Furthermore, the formation of the PCR product was monitored continuously during amplification by fluorescent dye (SYBR Green I dye), which binds to double-stranded DNA. The specificity of the reaction was, therefore, determined entirely by the specificity of the PCR primers, resulting in the presence of a single, specific PCR product (Figure 5.6), as the presence of double-stranded products generated during the PCR amplification would have been detected by the instrument. In addition, the use of glycerol and DMSO enhanced the optimal performance of PCR.

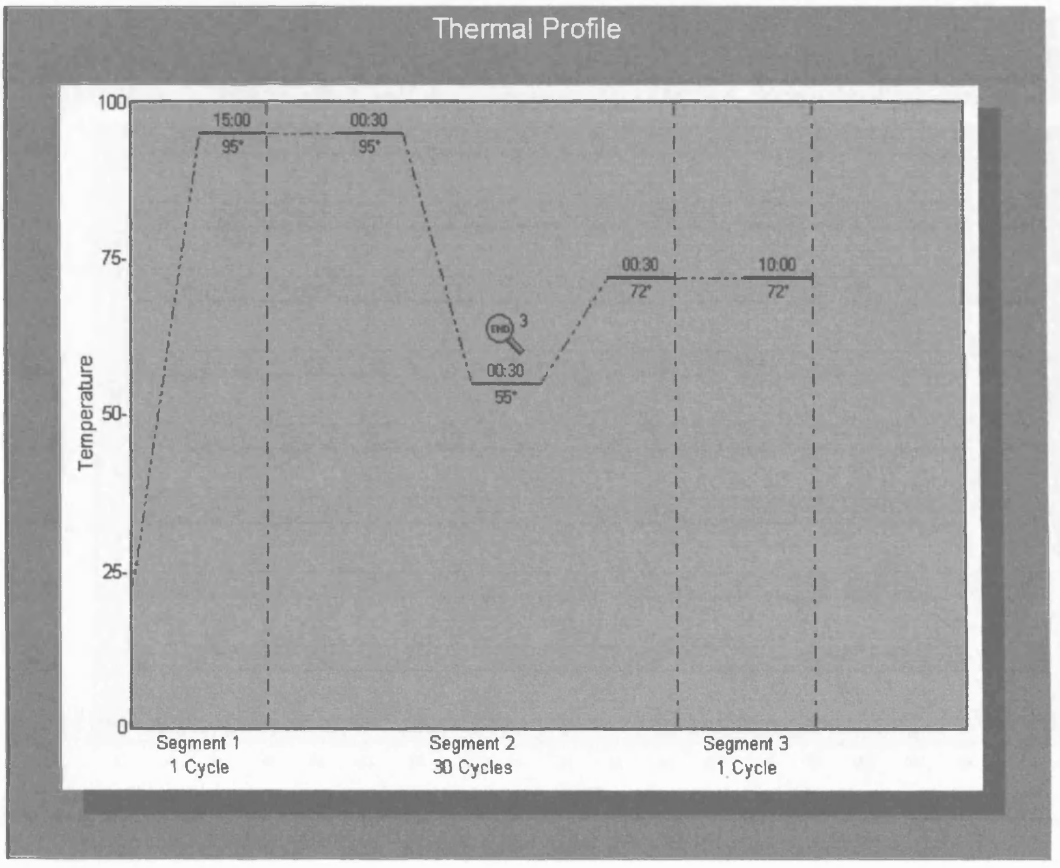


Figure 5.4: Thermal cycling profile. UVC-irradiated, *Bam*HI-digested human genomic DNA (50ng per PCR reaction) was amplified using SureStart^{R}Taq DNA polymerase in the MX4000® Multiplex Quantitative PCR System. The thermal cycling profile consisted of a 10-minute heat-inactivation step of SureStart^{R}Taq DNA polymerase (5 U/μl) at 95°C followed by thirty cycles each one consisted of 30 sec of DNA denaturation at 95°C, 30 sec of primer-template hybridisation at 55°C, and 30 sec of primer extension at 72°C, and a final extension of 10 minutes at 72°C.

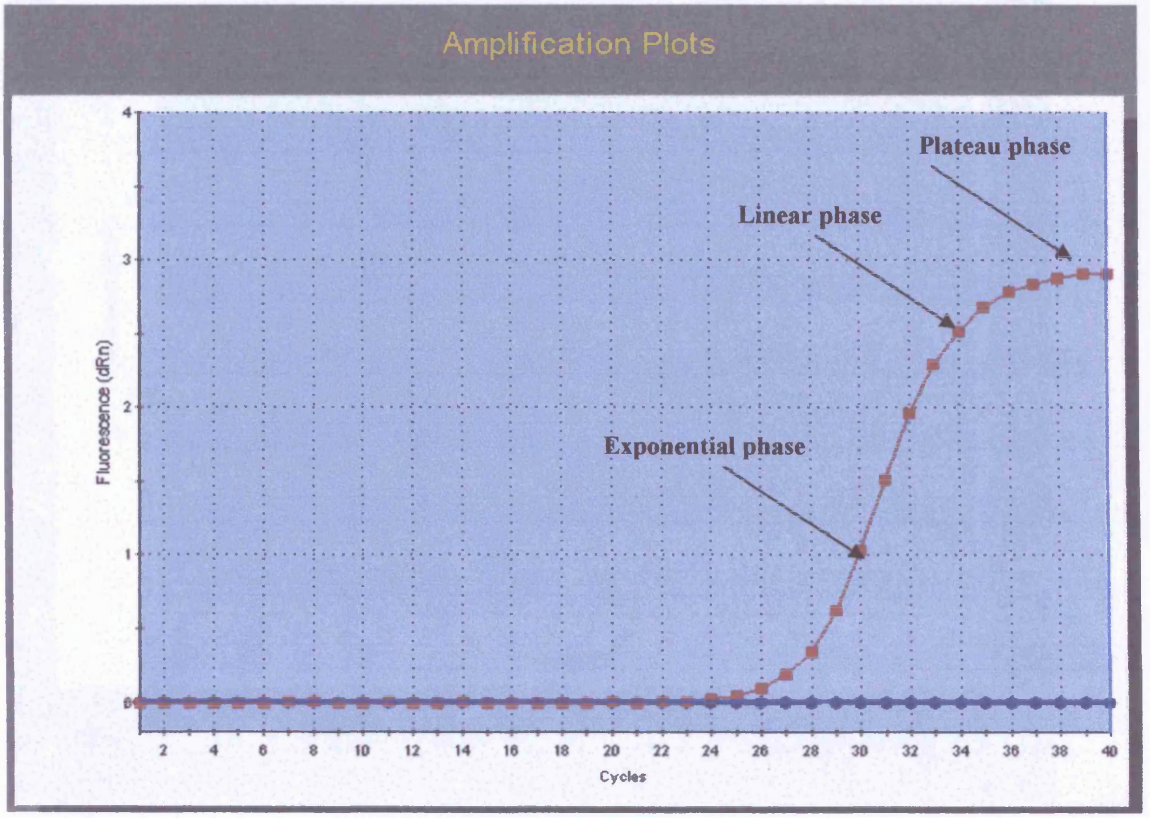


Figure 5.5: PCR amplification of human genomic DNA using Real-Time Quantitative PCR (QPCR). UVC-irradiated, *Bam*HI-digested genomic DNA (50ng) was amplified using real-time QPCR. The graph shows that amplification of human genomic DNA under the conditions described in Figure 5.4 was within the exponential phase of the reaction.

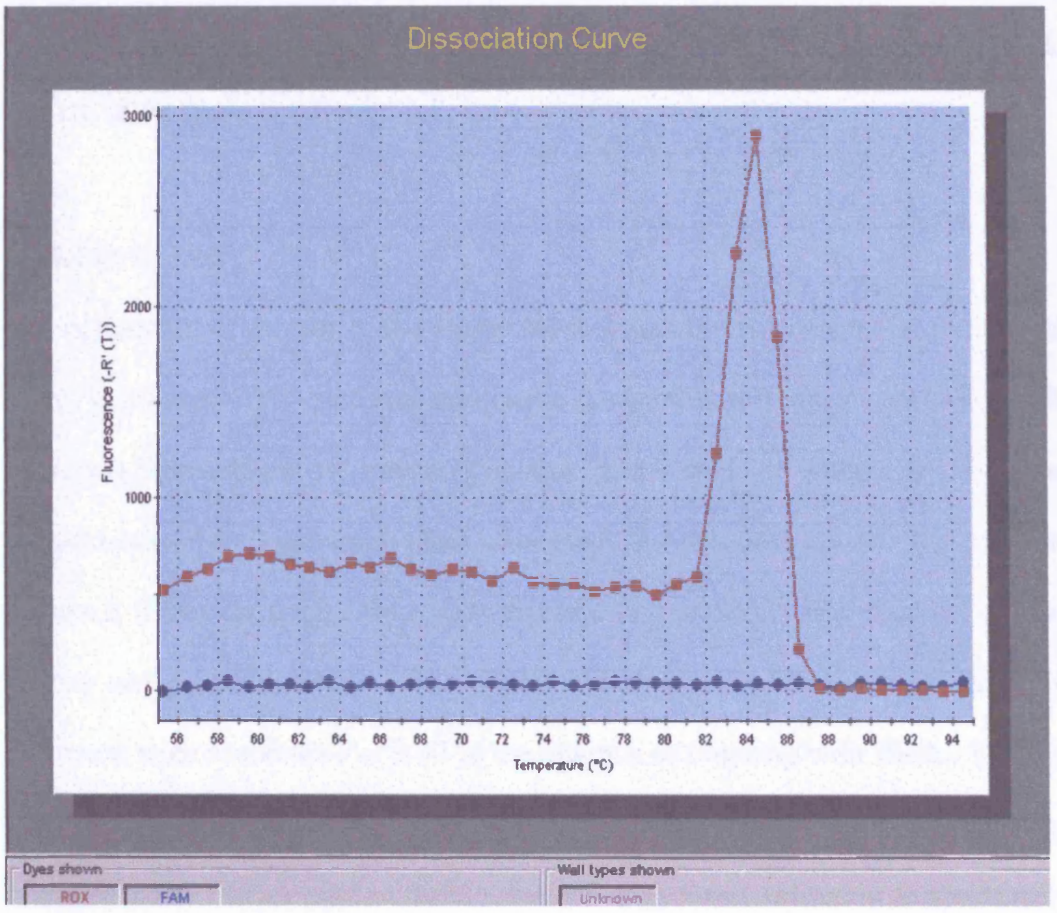


Figure 5.6: Dissociation curve using SYBR Green I fluorescence dye. 50ng of UVC-irradiated, *Bam*HI-digested genomic DNA were amplified using real-time QPCR as described above (Figure 5.4). The graph shows the specificity of the H-*ras* primers in amplification of human genomic DNA.

5.4.3 Establishment of the importance of protein A and Blotto blocker by (i) ELISA and (ii) ICPCR

5.4.3.1 (i) ELISA

The importance of protein A and Blotto blocker was investigated by ELISA and ICPCR assay. In ELISA, UVC-irradiated and native (non-irradiated) single-stranded calf thymus DNA was bound either to protein A coated ELISA wells or without it (PBS was used instead to avoid well variability) and were blocked either with Blotto blocker solution or without it (PBS was used). The results revealed that protein A was essential in antiserum binding giving an absorbance value of 0.60 at 492nm while blocking with Blotto blocker compared to an absorbance of 0.40 in the absence of blocking with Blotto. These results clearly indicate the efficiency of Blotto blocker in blocking any non-specific binding sites available in the ELISA plate, allowing thus, the polyclonal antiserum to specifically bind to UVC-irradiated DNA (Figure 5.7). The binding of antiserum to unirradiated DNA was insignificant irrespective of the presence of protein A or Blotto. A decrease in antiserum binding to UVC-irradiated DNA was detected in the absence of protein A from the ELISA plate irrespective of the blocking step giving absorbance values of 0.11 and 0.20 at 492nm following blocking without or with Blotto respectively (Figure 5.7). These results further support the importance of protein A in antiserum binding and the effectiveness of Blotto blocker in blocking non-specific sites.

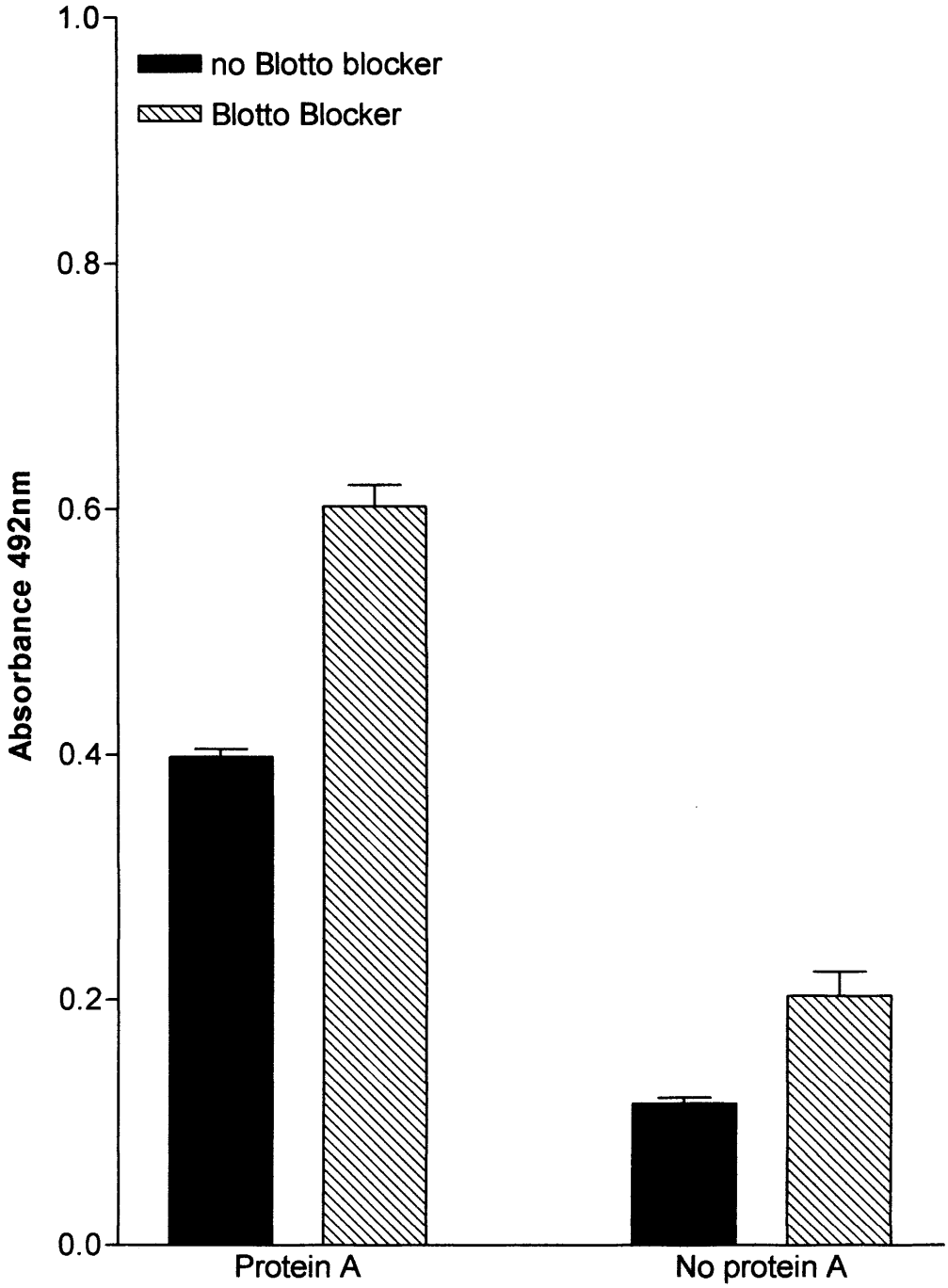


Figure 5.7: ELISA showing protein A and Blotto blocker efficiency. Calf thymus DNA (UVC-irradiated and non-irradiated DNA; 50μg/ml in PBS) was bound either to a protein A coated or without it ELISA plate and blocked either with Blotto blocker or without it. Values represent the mean (± S.D.) of three experiments.

5.4.3.2 (ii) ICPCR

A working volume of blocking (Blotto) and washing solutions (PBS and Tris-HCl) was established using different volumes of Blotto blocker (50-500µl per tube), PBS and Tris-HCl (100-500µl per tube) in the ICPCR with UVC-irradiated and unirradiated, *Bam*HI-digested human genomic DNA as templates. The results showed a volume of 500µl of Blotto to be effective in blocking any non-specific binding sites in the tube whereas lower volumes resulted in insufficient blocking and the presence of non-specific PCR amplification. That volume was used consequently in all the ICPCR experiments. Similarly, 500µl of PBS was found to be sufficient to remove any excess unbound antibody or antigen from the tube and 200µl of Tris-HCl resulted in elution of pure DNA into the tube for subsequent PCR amplification.

Having established a working volume for the blocking and washing solutions, UVC-irradiated and unirradiated, *Bam*HI-digested genomic DNA were assayed by ICPCR as described previously using 500µl Blotto for blocking the tubes, 500µl PBS for washing and 200µl Tris-HCl. Analysis of PCR products by agarose gel electrophoresis revealed that in the presence of protein A in the tube and blocking by Blotto specific PCR products were detected (Figure 5.8: 1st gel; lanes 2,4) compared to no PCR bands in the absence of protein A (Figure 5.8: 1st gel; lane 3) and non-specific amplification in the absence of Blotto (Figure 5.8: 2nd gel; lanes 5,6). These results clearly demonstrate the importance of protein A in antiserum binding. Protein A, a polypeptide isolated from *Staphylococcus aureus*, binds the Fc region of IgG molecules without interacting at the antigenic binding site, permitting thus, the formation of tertiary complexes consisting of antiserum, antigen and protein A. This is further supported by Brennand and Margison (1986), where the use of protein A in their experiment resulted in an increase in the amount of functional binding

of antiserum to the microtube surface. Furthermore, Blotto blocker was found to be effective in blocking any non-specific binding sites available in the tube compared to non-specific amplification observed in the tubes without blocking. The binding of antiserum to control, unirradiated tube was insignificant (Figure 5.8: 1st gel; lane 1) suggesting the specificity of polyclonal antiserum to recognise UVC-induced DNA damage. This specificity of the antiserum is further supported by the results of the experiment described below.

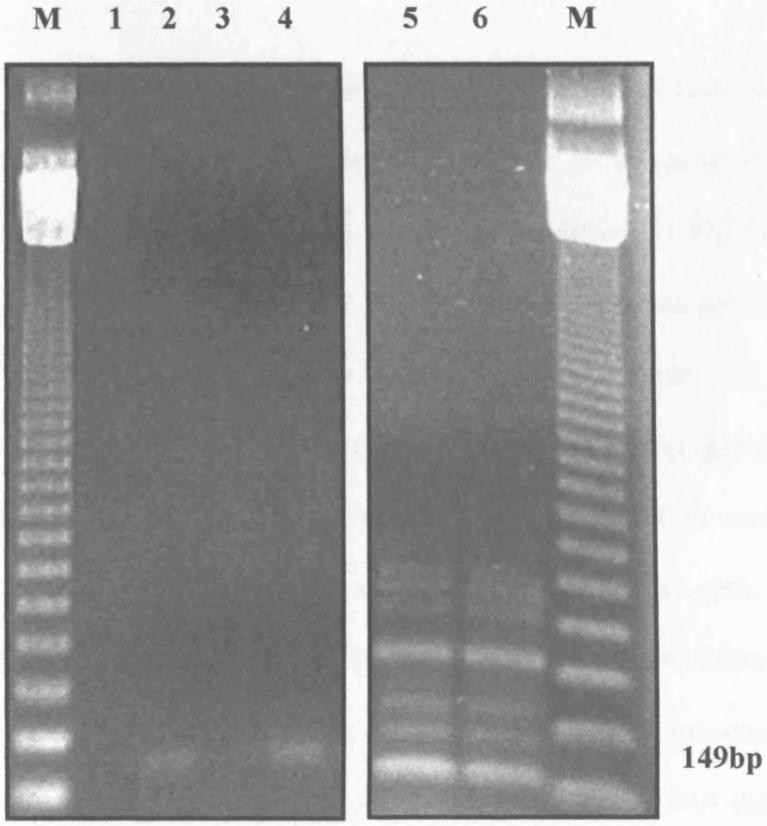


Figure 5.8: Detection of the efficiency of protein A and Blotto blocker by ICPCR. 50ng digests of amplified human genomic DNA samples were assayed by ICPCR after addition of either protein A or Blotto blocker. Lane 1 shows control, unirradiated, *Bam*HI-digested DNA with protein A and Blotto. Lane 3 shows UVC-irradiated (1.47mJ/cm^2), *Bam*HI-digested DNA (50ng) without protein A but with Blotto. Lanes 2 and 4 show 50ng of UVC-irradiated (1.47mJ/cm^2), *Bam*HI-digested DNA with protein A and Blotto. Lanes 5-6 show UVC-irradiated, *Bam*HI-digested DNA without Blotto but with protein A. 40µl PCR aliquots were used. M, 123bp DNA Ladder.

5.4.4 Establishment of the specificity of polyclonal antiserum to recognise UVC-induced DNA damage in the human H-*ras* gene by ICPCR assay

The specificity of the polyclonal antiserum IgG479 to recognise UVC-induced DNA damage in gene-specific sequences within the human H-*ras* proto-oncogene was established by ICPCR using unirradiated and UVC-irradiated (1.47KJ/m^2), *Bam*HI-digested human genomic DNA. Analysis of PCR products by agarose gel electrophoresis showed specific amplification of the 149bp fragment (Figure 9; lanes 4-6). Non-specific binding of antiserum to control, unirradiated DNA was not detected (Figure 9; lanes 1-2), clearly indicating the specificity and sensitivity of the antiserum to recognise UVC-induced DNA damage in discrete fragments in the human H-*ras* gene. Furthermore, although UVC-irradiated DNA was used in ICPCR, the PCR product of interest could not be detected (Figure 5.9; lane 3), suggesting the requirement of the antiserum attached to the surface of the tube in order to detect specific immunocapture of DNA damage in gene-specific sequences following UVC radiation.

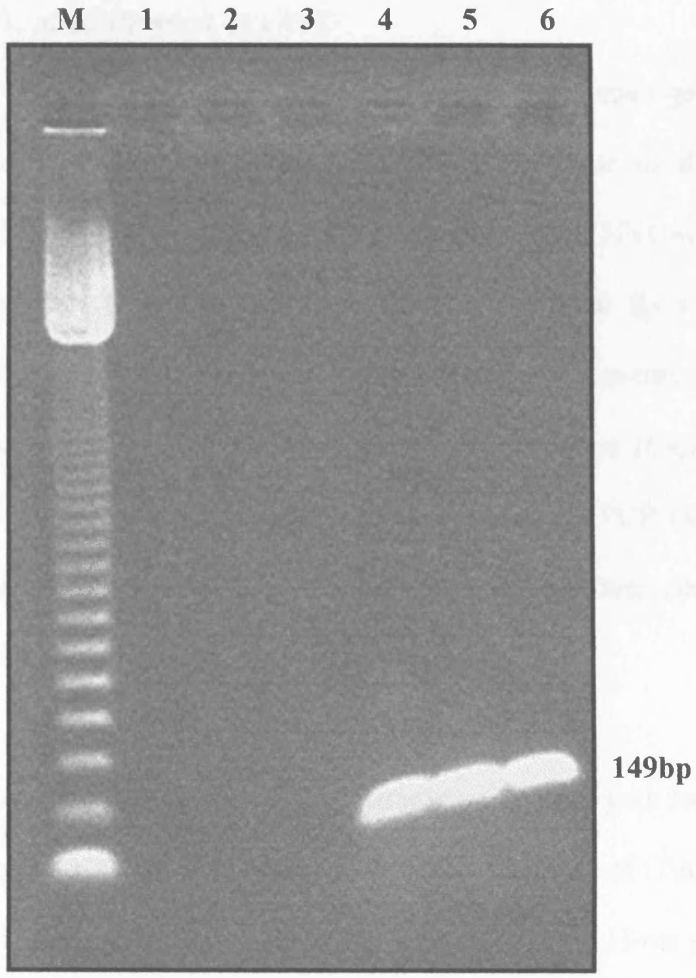


Figure 5.9: Detection of UVC-induced DNA damage in the human H-*ras* gene by polyclonal antiserum IgG479. 50ng digests of amplified human genomic DNA samples were assayed by ICPCR. Lanes 1-2 show control, unirradiated, *Bam*HI-digested human genomic DNA (50ng). Lanes 3-6 show 50ng of UVC-irradiated (1.47 KJ/cm^2), *Bam*HI-digested human genomic DNA without (lane 3) and with antiserum IgG479 (lanes 4-6). M, 123bp DNA Ladder.

5.4.5 Gene-specific quantification of T<>T

Quantification of DNA damage induced by UVC radiation in human genomic DNA was achieved using single-tube ICPCR assay, in a manner similar to that described by Denissenko *et al.* (1996), based upon the immunocapture of UVC-irradiated, *Bam*HI digested genomic DNA fragments by lesion-specific antiserum IgG479. Digestion of human genomic DNA with *Bam*HI restriction endonuclease was essential to quantitatively release the *ras* gene-containing segment prior to immunocapture (Capon *et al.*, 1983). Upon capture, the immuno-recovered template was amplified by PCR using gene-specific primers, which include amplification of a 149bp target region around codon 12 of exon 1 of the human H-*ras* proto-oncogene (Denissenko *et al.*, 1994).

PCR amplification of human genomic DNA, irradiated *in vitro* with two doses of UVC radiation (0.294 and 1.47KJ/m^2) showed specific immunocapture of UVC-irradiated DNA by the polyclonal antiserum IgG479. The 149bp PCR target of the H-*ras* gene was detected in both UVC-irradiated DNA samples (Figure 5.10; lanes 2-3). However, although the same amount (50ng) of human genomic DNA was used in each PCR, the intensity of the PCR signal was stronger in the DNA sample irradiated with a dose of 1.47KJ/m^2 than that with 0.294KJ/m^2 of UVC radiation. This suggests that the amount of the PCR product is proportional to the initial amount of damage as the higher the dose of UVC radiation the stronger the intensity of the PCR signal. The antiserum binding to control, unirradiated DNA sample was insignificant (Figure 5.10; lane 1), due to the specificity of the polyclonal antiserum to recognise DNA damage following exposure to UVC radiation.

Serially diluted *Bam*HI-digests of unirradiated human genomic DNA of known concentration (0-5ng) were used as standards in the quantification of PCR products by

comparing the band intensity of the 149bp PCR product (Figure 5.10; lanes 4-7). Observing the Figure, the intensity of the PCR band was stronger when a higher amount (5ng) of template DNA (unirradiated, *Bam*HI-digested human genomic DNA; lane 6) was used in PCR compared with those with a lower amount of template DNA (lanes 4, 5 and 7). However, an even stronger intensity in the PCR signal was seen when a higher amount of template DNA (50ng of UVC-irradiated, *Bam*HI-digested human genomic DNA; lane 3) was used in PCR, implying that the amount of the PCR product is proportional to the initial amount of template DNA.

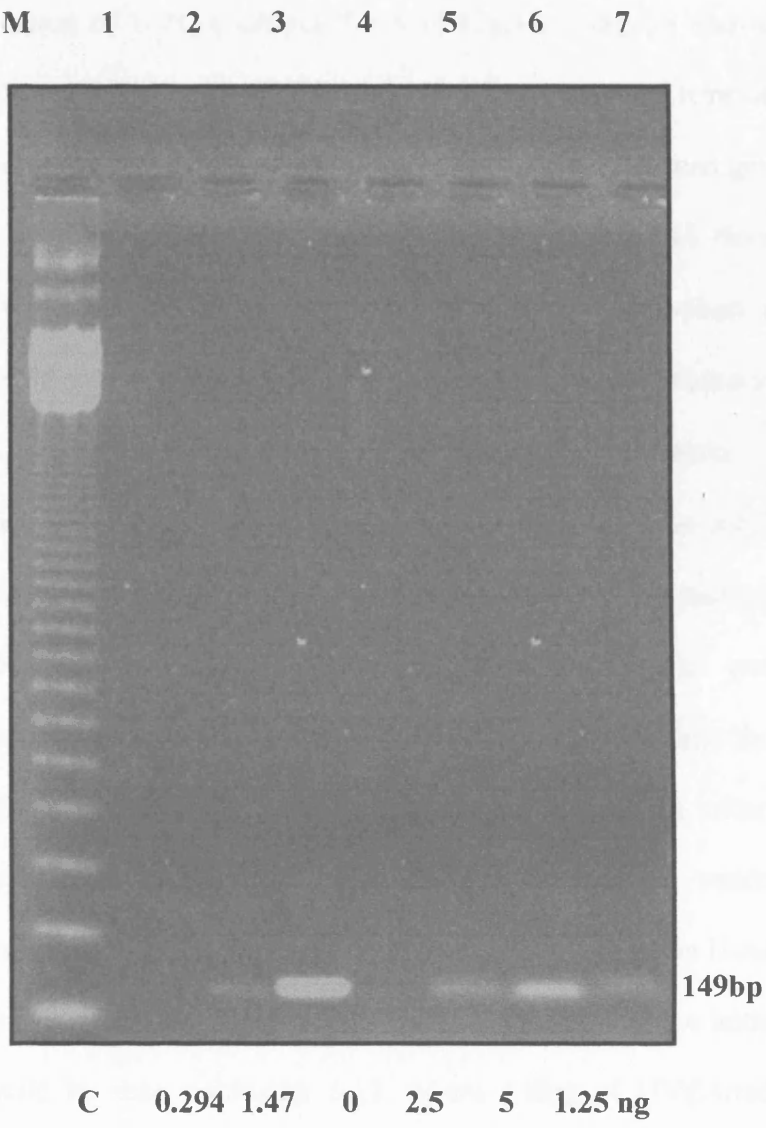


Figure 5.10: Representative gel showing PCR amplification of UVC-irradiated human genomic DNA. Human genomic DNA samples (1mg/ml) were irradiated with two doses of UVC radiation (0.294 and 1.47KJ/m^2), *Bam*HI digested and 50ng digests were assayed by ICPCR using polyclonal antiserum IgG479. Lane 1 shows control, unirradiated human genomic DNA. Lanes 2-3 show UVC-irradiated genomic DNA (0.294KJ/m^2; lane 2 and 1.47KJ/m^2; lane 3). Lanes 4-7 show PCR standards (0-5 ng unirradiated, *Bam*HI-digested human genomic DNA) run in parallel. 40µl PCR aliquots were used. M, 123bp DNA Ladder.

Similarly, amplification of UVC-irradiated DNA (1.47KJ/m^2) digests showed a highly significant ($p<0.0001$), linear relationship between the initial amount of template DNA and the resulting amount of PCR product over the range 10 –100 ng of human genomic DNA ($r^2=0.99$; Figure 5.11), as the higher the amount of PCR amplified DNA the stronger the intensity of the PCR signal. This linear response confirms that the values obtained by ICPCR were derived from the measurement of the original template concentration of lesion (Hochleitner *et al.*, 1991, Kalinowski *et al.*, 1992) and not from interferences with *Bam*HI restriction endonuclease, or *Taq* polymerase. The primers used in the amplification of genomic DNA were designed by Denissenko and colleagues (1994) in such a way so that the amplified DNA region to be much smaller than the restricted gene fragment precipitated by the polyclonal antiserum IgG479. This target selection and the doses used in the UVC-irradiation of genomic DNA caused no interference with either of the two enzymes (*Taq* polymerase and *Bam*HI restriction endonuclease) resulting in the immunocapture and quantification of a 149bp fragment within the human H-*ras* gene. This proportional relationship between the amount of the PCR product and the initial amount of template DNA could be seen on Figure 5.12, where 100ng of UVC-irradiated DNA (1.47KJ/m^2) digests resulted in an intense PCR product (lanes 2-4) compared with PCR digests starting with a lower amount (25ng) of template DNA (lanes 5-7). Non-specific immunocapture could not be seen with an excess amount of control, unmodified DNA.

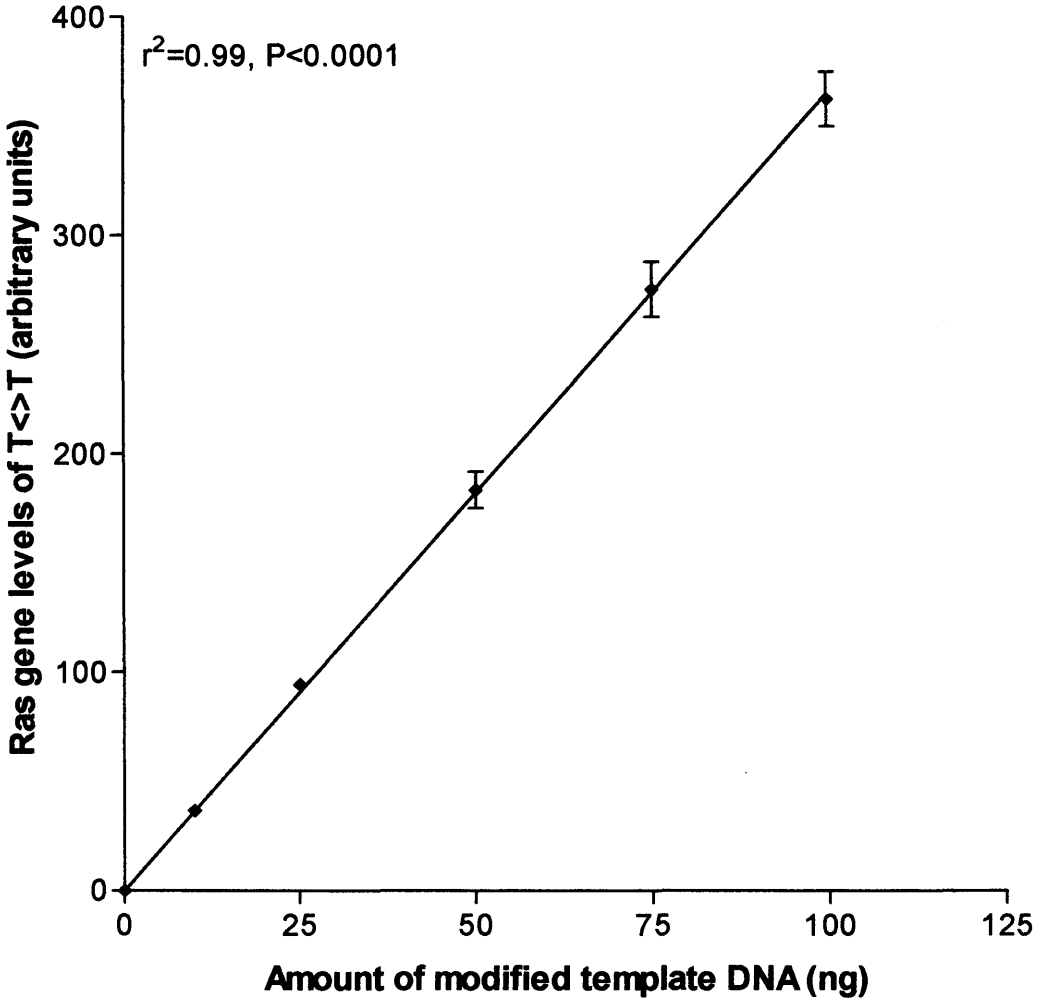


Figure 5.11: Quantification of UVC-induced DNA damage in human genomic DNA. Different amounts (0-100ng) of UVC irradiated (1.47KJ/m^2) were digested with *Bam*HI restriction endonuclease and immunodetected with IgG479 antibody. The intensity of each individual band was detected by densitometry and plotted against increasing amount of template DNA by first order linear regression (Sigmaplot). Values represent the mean (± SEM) of three experiments per concentration.

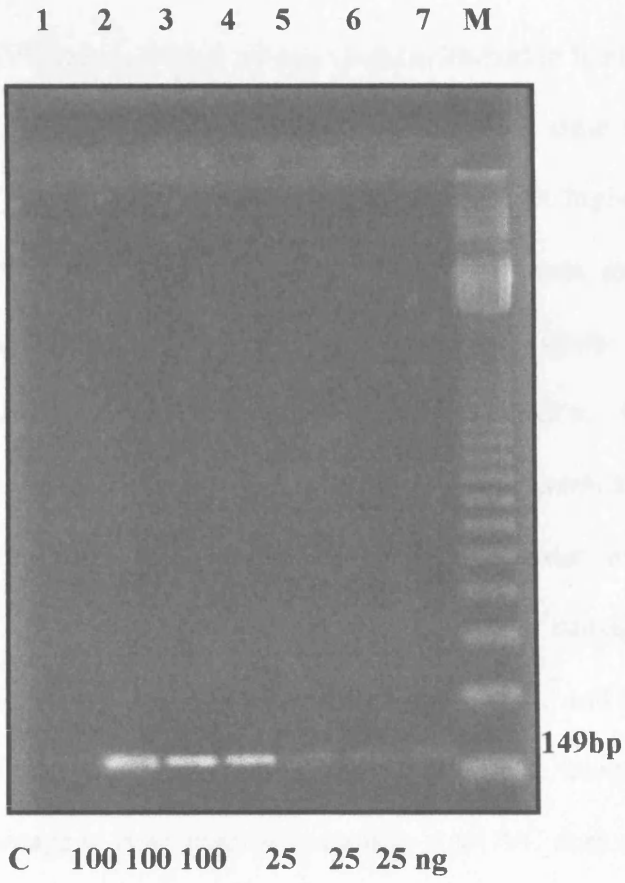


Figure 5.12: Specific immunodetection of UVC-irradiated human genomic DNA by ICPCR assay. Human genomic DNA samples (1mg/ml) were UVC-irradiated (1.47KJ/m^2), digested with *Bam*HI restriction endonuclease and different amounts of template DNA were assayed by ICPCR. Lane 1 shows control, unirradiated human genomic DNA (100ng). Lanes 2-7 show UVC-irradiated genomic DNA (lanes 2-4: 100ng; lanes 4-7: 25ng). PCR products (40µl) were analysed on a 2% agarose gel. M, 123bp DNA Ladder.

The quantification of UVC-induced DNA damage was performed in human genomic DNA after exposure to 0-3.48 KJ/m^2 of UVC radiation. Although the same amount of human genomic DNA (50 ng) used for each PCR, DNA samples with higher doses of UVC radiation were preferentially captured by the lesion-specific antiserum, resulting in a UVC-dose dependent increase in the intensity of the PCR signal (Figure 5.13). This linear response was present even at low doses of UVC radiation (0-1.47KJ/m^2; Figure 5.14 and 0-0.05KJ/m^2; Figure 5.15), confirming that the amount of the PCR product is proportional to the initial amount of damage (UVC radiation). Moreover, a greater linearity (r^2 = 0.98) between the amount of the PCR product and the initial amount of damage was detected at lower doses (0-0.05KJ/m^2) compared to higher doses (0-3.48KJ/m^2 and 0-1.47KJ/m^2) with an r^2 = 0.94 and 0.97 respectively. ICPCR assay was established, therefore, to be able to detect UVC-induced damage in gene-specific sequences at a UVC dose of as low as $3J/m^2$. The antibody binding to control, unirradiated sample was insignificant, clearly indicating the specificity of immunodetection for UVC-irradiated DNA.

However, ICPCR response appeared to approach a plateau level at the highest dose of UVC radiation (3.48KJ/m^2) (Figure 5.13). Previous work by Wani and Arezina (1991) showed a saturation level in the response of their immunodetection assay at higher UVC doses. Similarly, Denissenko *et al.* (1994) reported a plateau level in the immuno-slot-blot response at *anti*-BPDE genotoxin concentrations above 0.5μM for 25ng of genomic DNA. By decreasing the amount of DNA in ICPCR assay, a linear response in the quantification of UVC-induced DNA damage could be achieved. Indeed, application of ICPCR assay *in vivo* resulted in a linear induction of T<>T in UVB-irradiated human keratinocytes when a smaller amount (25ng instead of 50ng) of template DNA was used in ICPCR experiments. The plateau level in ICPCR assay was probably caused by limitations in antibody binding

given that the antibody had bound a maximum number of sites for lesions available in the tube and not from a decrease in the induction of T<>T at high doses of UVB radiation. Thus, the sensitivity of immunodetection could be improved by the use of larger amounts of template DNA in the ICPCR assay, previously described in Denissenko *et al.* (1994).

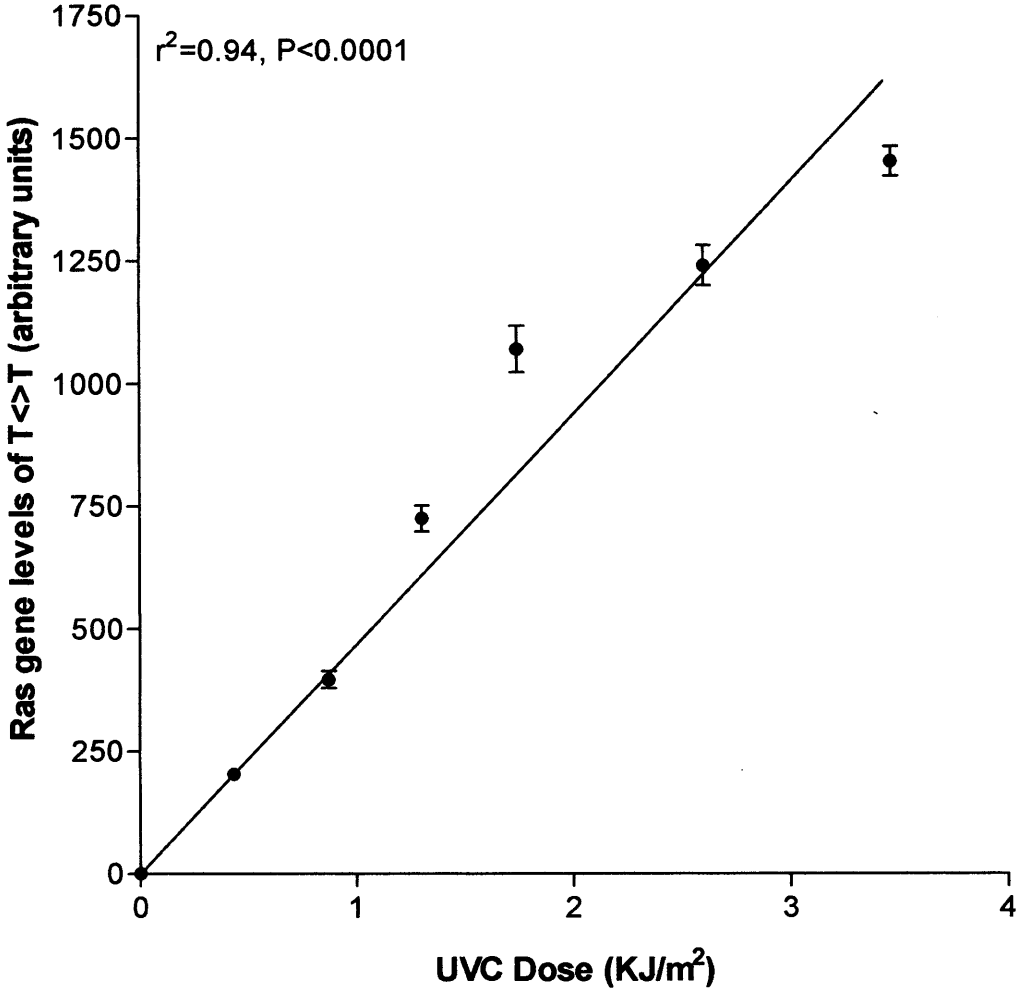


Figure 5.13: ICPCR quantification of T<>T in human H-*ras* gene following UVC radiation. Human genomic DNA samples (1mg/ml) were irradiated with increasing doses of UVC radiation (0 to 3.48KJ/m^2), digested with *Bam*HI enzyme, and 50ng digests were assayed by ICPCR. The relative peak intensities were determined for each band and plotted against corresponding doses of UVC radiation by first order linear regression (Sigmaplot). Data represents the mean (±SEM) of three experiments per UVC dose.

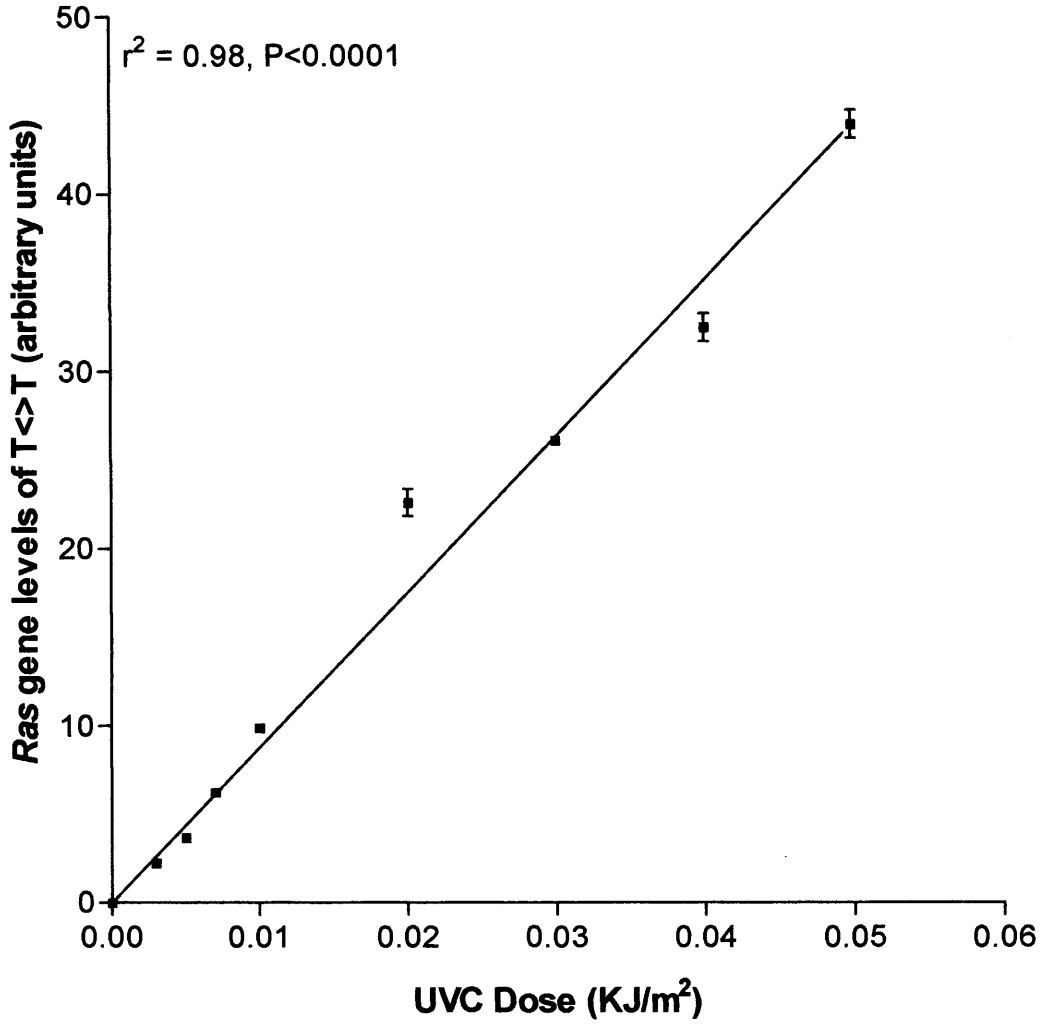


Figure 5.15: Establishment of the sensitivity of ICPCR in the detection of T<>T in human H-*ras* gene following UVC radiation. Human genomic DNA samples (1mg/ml) were irradiated with increasing doses of UVC radiation (0-0.05 KJ/m^2), digested with *Bam*HI restriction endonuclease and 50ng digests were assayed by ICPCR. The intensity of the individual bands was determined by densitometry and the levels of T<>T in the H-*ras* gene was plotted against corresponding doses of UVC-radiation by first order linear regression (Sigmaplot). Data represents the mean (±SEM) of three experiments per UVC dose.

5.4.6 Global genome quantification of UVC-induced T<>T by competitive ELISA

Quantification of DNA damage induced by different doses of UVC radiation (0-3.48KJ/m^2) in the human genomic DNA was achieved using competitive ELISA as described earlier. In competition ELISA, a concentration of 0.195μg/ml of UVC-irradiated human genomic DNA was found to be the best inhibitor for antiserum binding with an IC_{50} of 2.95μg/ml (Figure 5.16). The IC_{50} is an index of the antigenicity of a compound where the lower the value the better the inhibitor. Higher or lower concentrations of UVC-irradiated human genomic DNA showed little inhibition with the polyclonal antiserum IgG479 (IC50>100μg/ml). Thus, 0.195μg/ml of UVC-irradiated human genomic DNA was used in the comparison of gene- and global-level measurements of cyclobutane thymine dimers.

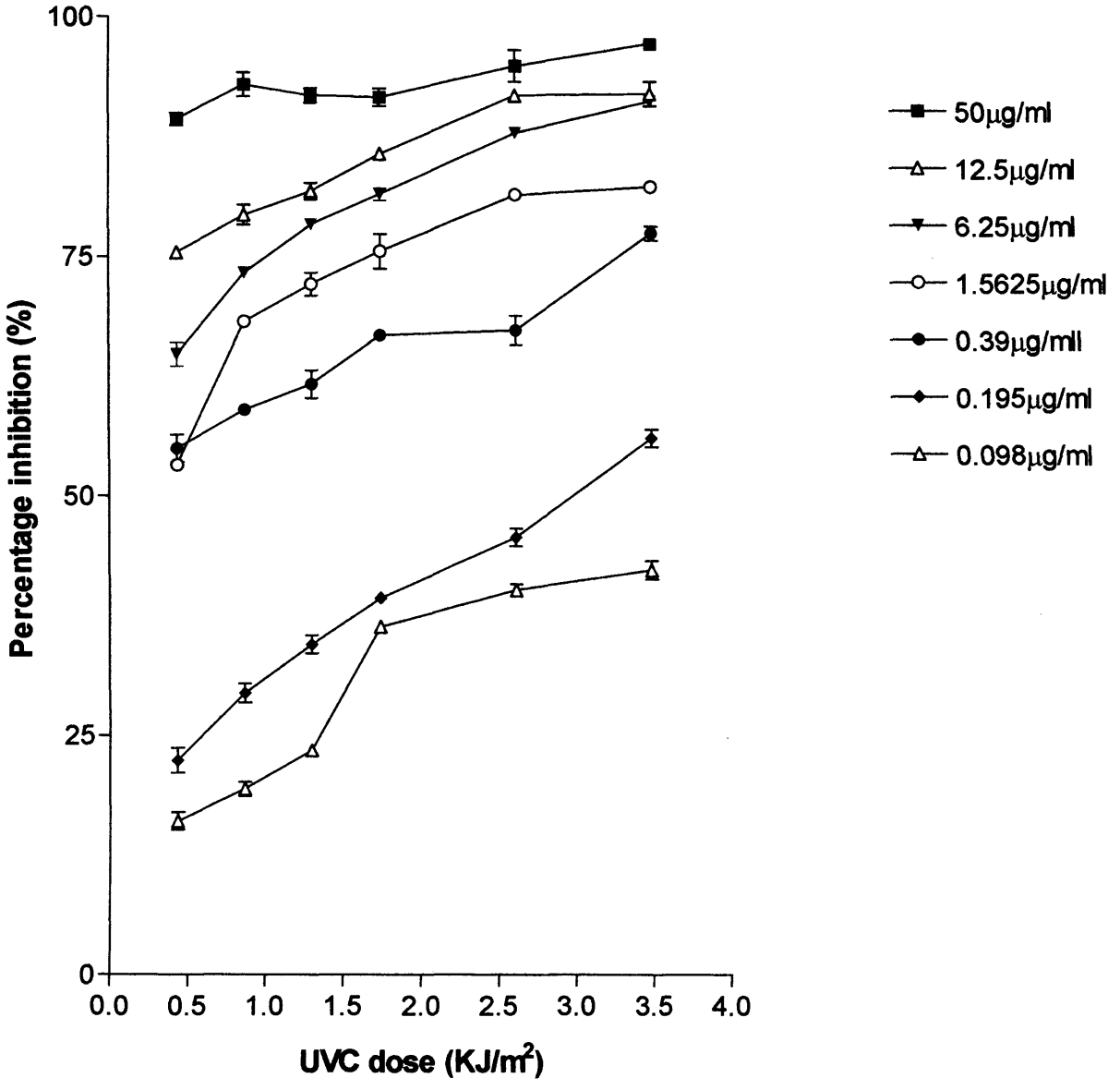


Figure 5.16: Global measurement of T<>T following different doses of UVC radiation by ELISA assay. Human genomic DNA was exposed to different doses of UVC radiation (0-3.48KJ/m^2) and assayed by competitive ELISA. Values represent the mean (±S.D.) of three determinations per concentration.

5.4.7 Quantification of T<>T in UVC-irradiated DNA by GC-MS

Stable-isotope mass spectrometry was used to determine accurately the levels of *cis-syn* T<>T produced in UVC-irradiated DNA following a dose range (0-3.48 KJ/m^2), as described previously (Podmore *et al.*, 1996). The graph of dose versus yield demonstrated linear-dose-yield relationship (Figure 5.17). This agrees with previous observations in calf thymus DNA using ^{32}P-postlabelling (Bycov *et al.*, 1995). GC-MS permitted absolute quantification along with positive identification of T<>T lesions within UVC-irradiated DNA. GC-MS sensitivity was determined to be 20-50 fmol (0.02-0.05 nmol/mg DNA), or 6-15 lesions per 10^6 bases (Podmore *et al.*, 1996).

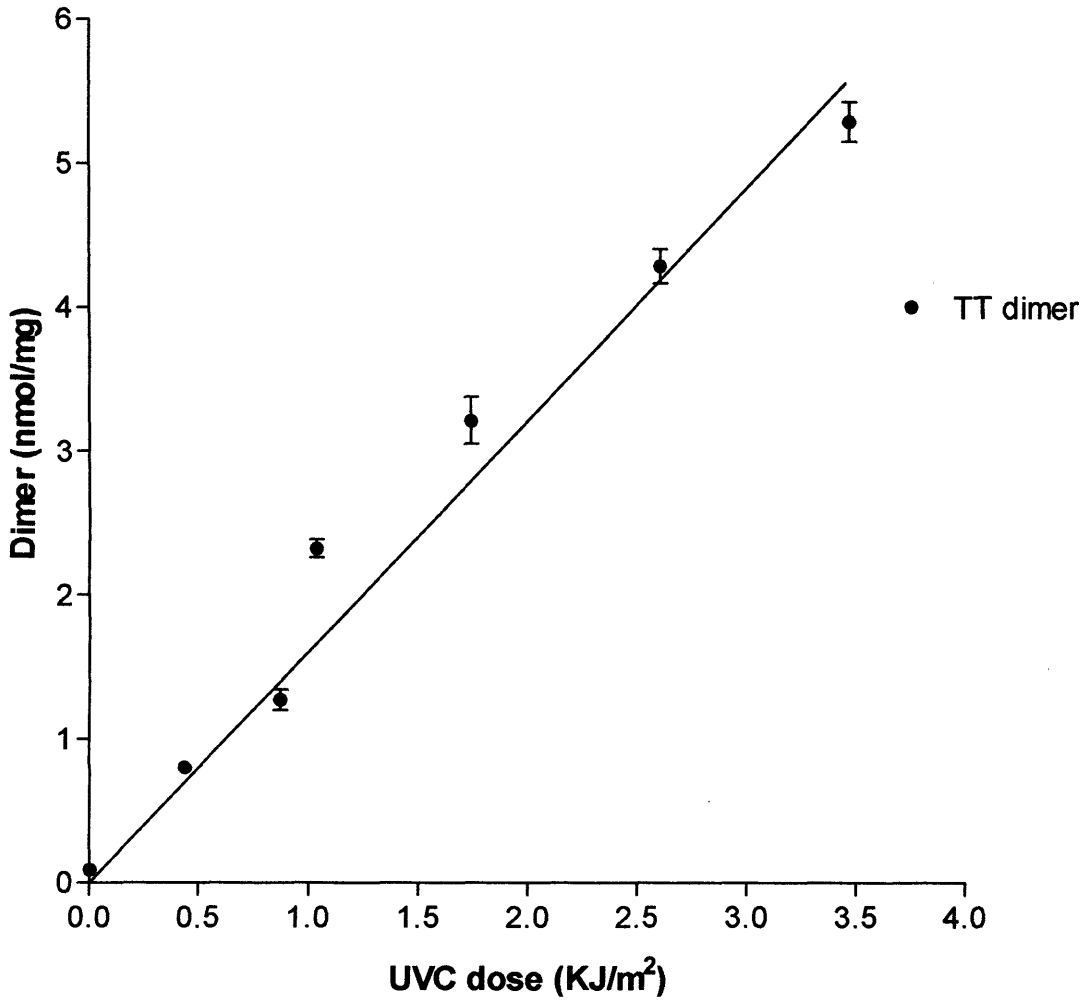


Figure 5.17: Induction of *cis-syn* cyclobutane thymine dimers (T<>T) in UVC-irradiated DNA. Each point is the mean (±S.D.) value of three independent autosampler injections.

5.4.8 Comparison of gene- and global damage assessments

Human genomic DNA was UVC-irradiated (0-3.48KJ/m^2), *Bam*HI-digested and assayed by ICPCR as previously described. The results obtained from ICPCR assay (Figure 5.13) were compared to those obtained by competitive ELISA using 0.195μg/ml UVC-irradiated genomic DNA (Figure 5.16) in an attempt to correlate measures of T<>T induction in gene-specific sequences within the human H-*ras* proto-oncogene and in the global genome following UVC radiation. Comparison of the results from competitive ELISA with those obtained from ICPCR showed a significant ($P<0.0001$) correlation ($r^2 = 0.91$) between measures of T<>T in the H-*ras* gene and the global genome (Figure 5.18). The induction of UVC-induced T<>T within gene-specific sequences in the human H-*ras* gene and the total genome occurred in a linear dose-responsive manner since a saturation in antibody binding for UVC doses as high as 3.48KJ/m^2 was not detected until after the highest UVC dose (3.48KJ/m^2) as previously shown in Chapter 3.

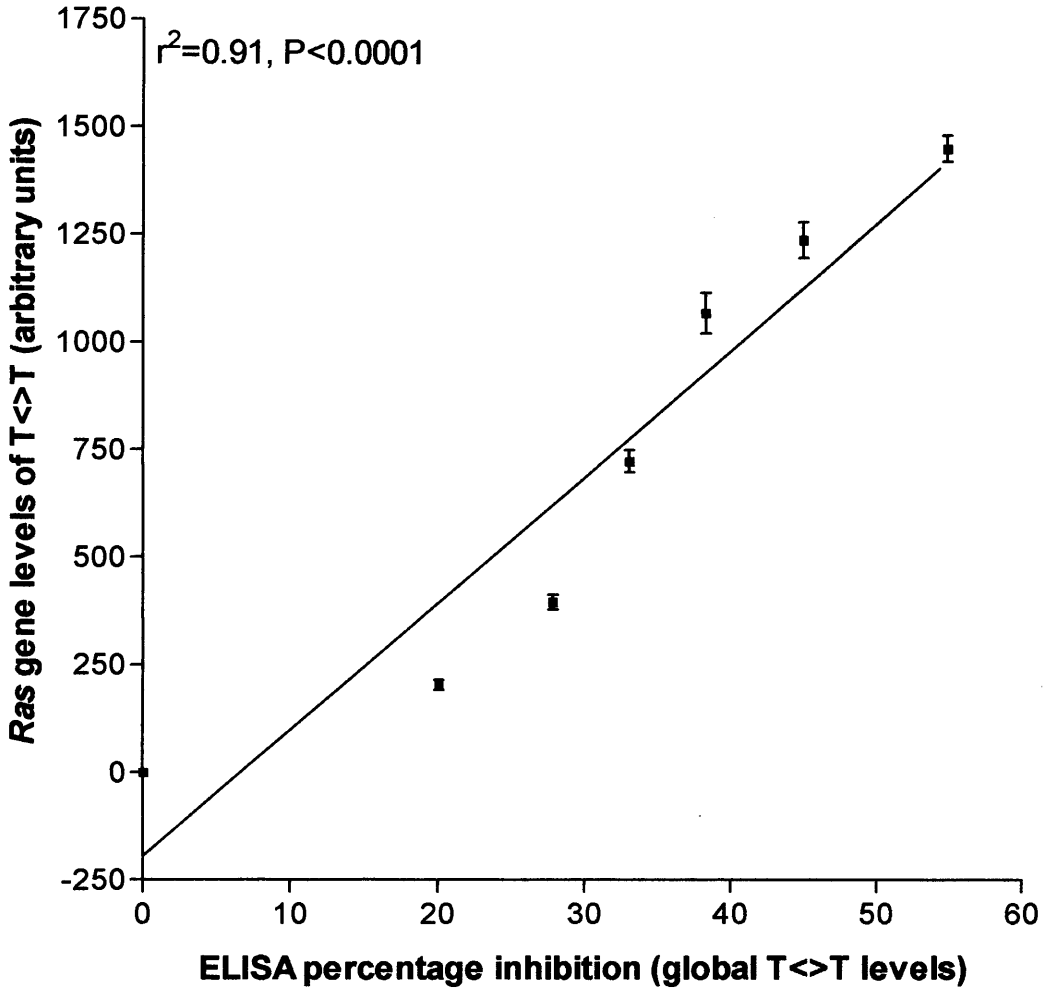


Figure 5.18: Comparison of gene- and global genome assessments of T<>T following UVC radiation. Human genomic DNA was UVC-irradiated (0-3.48KJ/m^2) and quantified by ICPCR. Levels of T<>T induced by UVC radiation in the human H-*ras* gene were compared to levels in the global genome determined by ELISA. The levels of T<>T in the H-*ras* gene were plotted against those in the global genome by first order linear regression (Sigmaplot). Data represents the mean (±SEM) of three experiments per UVC dose.

Similarly, comparison of the T<>T measurements in the global genome induced by different doses of UVC radiation (0-3.48KJ/m^2) as it was determined by GC-MS (Podmore *et al.*, 1996; Figure 5.17) with the T<>T measurements obtained by ICPCR in the H-*ras* gene, revealed again a significant ($P<0.0001$) correlation ($r^2 = 0.97$) between H-*ras* gene measurements of T<>T and those in the overall genome (Figure 5.19).

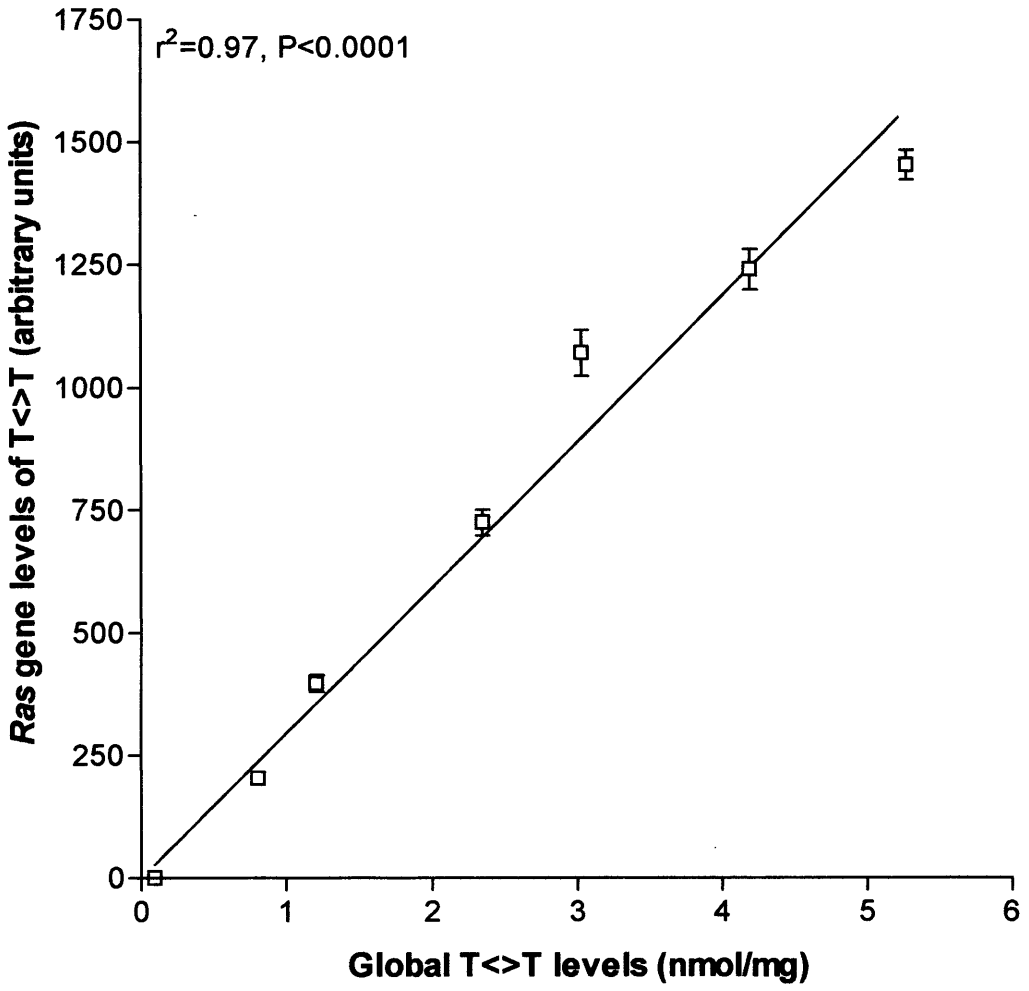


Figure 5.19: Comparison of gene- and global genome assessments of T<>T following UVC radiation. Human genomic DNA was exposed to different doses of UVC radiation (0-3.48KJ/m^2), analysed by ICPCR and compare to global levels determined by GC-MS. The levels of T<>T in the H-*ras* gene were plotted against those in the global genome by first order linear regression (Sigmaplot). Data represents the mean (±SEM) of three experiments per UVC dose.

5.5 DISCUSSION

Heterogeneity of DNA damage and its repair has been established in different prokaryotic (Mellon and Hanawalt, 1989) and eukaryotic (Bohr *et al.*, 1985) cellular systems, enabling scientists to look for better methods in the detection and quantification of DNA damage and its repair in individual genes. The issues of quantification of DNA damage and the sensitivity of its detection are important ones, as further information on DNA damage and repair might be obtained. Antibody-based assays have been successfully employed to quantify DNA base modifications induced by various genotoxic agents (Wani and D'Ambrosio, 1987; Wani and Arezina, 1991; Denissenko *et al.*, 1994, 1996a).

In this study, a sensitive and semi-quantitative approach was developed to assess DNA damage within specific-gene sequences in the human H-*ras* gene following UVC radiation. The ICPCR assay relies upon proportionality between the amount of PCR product and the initial amount of damage, previously demonstrated by Denissenko *et al.* (1994). The linearity between the dose of UVC-radiation and resulting amount of PCR product, demonstrated that the IgG479 is able to specifically bind T<>T contained within H-*ras* gene fragments derived from UVC-irradiated genomic DNA. This is essential to the quantitative measurement of gene-specific DNA damage and repair (Bohr, 1991). ICPCR assay was demonstrated to be able to detect UVC-induced damage in gene-specific sequences at a UVC dose of as low as $3J/m^2$. Although, such a dose might still be lethal for cells, as a lethal dose of UVC radiation is about $2.5J/m^2$ (Van Houten *et al.* 2000), it does approximate to doses used by other, similar, techniques such as LMPCR ($10J/m^2$; Gao *et al.*, 1994) and only a 0.5-fold higher than the QPCR assay where Van Houten *et al.* (2000) described the use of a long PCR to measure doses of UVC radiation down to $2.5J/m^2$. A relatively high dose ($24KJ/m^2$) of UVC radiation has been used in the measurement of

DNA repair in 147-440bp fragments *in vivo* (Govan *et al.*, 1990). On the contrary, a low dose of UVC radiation (2.5KJ/m^2) was used in the measurement of gene-specific repair in human cells (Van Houten *et al.*, 2000) but in PCR fragments as long as 24 kb. Similarly, Wani and Arezina (1991) demonstrated the measurement of DNA damage and repair within larger fragments (>5kb length) of plasmid DNA by UVC doses as low as 1 to 2 J/m^2.

PCR primers used in this study were reported to amplify the 149bp target region in a 6.4kbp H-*ras* gene fragment (Denissenko *et al.*, 1994). Only nanogram amounts of DNA were required to achieve specific immunocapture and quantification. In addition, amplification was limited only by template availability, as the amount of the PCR product was shown to be proportional to the initial amount of template DNA. The results obtained by ICPCR assay agreed with those obtained from the immunocapture of genomic DNA from human fibroblasts, treated *in vivo* with *anti* BPDE genotoxin, by BP1 antibody (Denissenko *et al.*, 1994), where a linear relationship was shown for the initial amount of template DNA and the resulting amount of PCR product within 1-25ng for genomic DNA.

Replicate experiments and analysis of PCR standards run in parallel in each PCR were performed in order to establish the reproducibility of the assay. The effective amplification of 149bp fragment in each PCR and the non-existence of primer dimer formation made the use of the PCR feasible without the requirement of an internal standard, essential in relative analysis. The thermal cycling profile (30 cycles) used in ICPCR for amplification of specific-gene sequences within the human H-*ras* proto-oncogene was found to be sufficient for reproducible amplification of small amounts (nanograms) of template DNA.

The conditions used in the amplification of the target DNA template where within the exponential phase of the reaction as determined by real-time QPCR.

A highly significant, linear relationship was established from the correlation of measures of T<>T dimers in gene-specific segments using ICPCR assay and in total genome using both GC-MS and competitive ELISA, indicating that the induction of T<>T in gene-specific sequences is proportional to the induction of lesions in the global genome. Despite using the same antiserum for both the ICPCR and ELISA assays, a marginally better correlation was noted between ICPCR and GC-MS, than ICPCR and ELISA. This may be due to the fragmentation or hydrolysis of DNA utilised in ICPCR and GC-MS, respectively, allowing greater access to the lesions, whereas stearic hindrance may impede the competitive ELISA. Upon initial analysis, this result was unexpected, given that the distribution of damage throughout the genome is non-random, as some regions of the genome are more sensitive or exposed to damaging agents than others, and the preferential removal of DNA lesions from transcriptionally active genes over inactive regions. Similarly, within their cellular model, Denissenko *et al.* (1994) noted good agreement between gene and global level repair rates. However, as the comparisons were performed using extracted DNA, in the absence of repair enzymes, or nucleosomal proteins, the distribution of damage is likely to be much more homogeneous, which is supported by the similar responses noted with both GC-MS and competitive ELISA. Despite the findings of Denissenko *et al.* (1994), it would be expected that comparative analysis of T<>T in DNA derived from cells irradiated *in situ*, and following a period of repair, would not show the same agreement between the two methodological approaches, reiterating the potential importance of gene-specific assays.

CHAPTER 6: INVESTIGATION OF UVB-INDUCED DNA DAMAGE AND REPAIR IN HUMAN KERATINOCYTES BY IMMUNO-COUPLED PCR

CHAPTER 6

INVESTIGATION OF UVB-INDUCED DNA DAMAGE AND REPAIR IN HUMAN KERATINOCYTES BY IMMUNO-COUPLED PCR

6.1 INTRODUCTION

The distribution of DNA damage and repair in several biological systems was shown to occur in a non-random manner throughout the genome due to site-specific damage induction, sequence context, chromatin structure and preferential repair of actively transcribed genes *versus* inactive regions or the overall genome (Bohr *et al.*, 1985, 1987; Mellon and Hanawalt, 1989; Hanawalt, 1989). Thus, the development of methods for the measurement of DNA damage and repair in specific genes is important as further information on the biological effects of various genotoxins might be obtained. Antibodies directed against carcinogen-DNA base adducts were used in the detection of DNA damage and repair within distinct target DNA fragments employing immuno-coupled PCR (ICPCR) assay (Denissenko *et al.*, 1994; 1996) and immunoaffinity-quantitative PCR (Hochleitner *et al.*, 1991; Thomale *et al*; 1994). ICPCR assay has been previously applied in the quantitative assessment of UVC-induced DNA damage in gene-specific sequences within the human H-*ras* proto-oncogene by polyclonal antiserum IgG479 (previously described in Chapter 5).

6.2 AIM

The aim of the work presented in this chapter was (i) the application of ICPCR assay to the detection and quantification of UVB-induced cyclobutane thymine dimers (T<>T) and their repair in the spontaneously, immortalised human keratinocyte cell line (HaCaT) and

(ii) comparison of measures of T<>T induction and repair in gene-specific sequences and the global genome of HaCaT cells following exposure to UVB radiation. This was a logical advance of the procedure over the preceding chapter on naked DNA.

6.3 METHODS

6.3.1 Immuno-coupled PCR (ICPCR) assay

Quantification of DNA damage and repair in UVB-irradiated HaCaT cells was performed by ICPCR assay in a manner similar to that described in the general protocol (Section 2.14). 50ng of unirradiated or UVB-irradiated, *Bam*HI-digested genomic DNA in PBS containing 0.5M NaCl was added to the tubes. In addition, varying amounts (0-100ng) of UVB-irradiated (0.8KJ/m^2), *Bam*HI-digested genomic DNA were assayed by ICPCR assay as described earlier (Section 2.14).

6.3.2 PCR amplification of genomic DNA

Genomic DNA samples (UVB-irradiated, *Bam*HI digested genomic DNA) isolated from human keratinocytes were PCR amplified as previously described (Section 2.11). PCR positive (unirradiated, *Bam*HI digested DNA samples) and negative controls (no DNA just PCR reagents) were PCR amplified in the same way and included in each PCR. Analysis and quantification of PCR products was performed as described in Section 2.12. The band intensities were determined by scanning the gels with a laser scanner and integrating the data for peak areas with computer assisted software (QuantiScan BIOSOFT^R software, version 2.1). Genomic DNA samples (unirradiated, *Bam*HI-digested; 0-5ng), which were PCR amplified alongside the test DNA samples and run in parallel, were used as standards in the quantification of PCR products by comparing the band intensity of the 149bp PCR

product. Statistical analyses were performed using GraphPad Prism, version 2.01 (GraphPad Software, San Diego, CA).

6.3.3 Investigation of the specificity of antiserum IgG479 to recognise UVB-induced DNA damage within gene-specific sequences in the human H-*ras* gene by ICPCR

Following overnight incubation at 4°C, protein A coated tubes (100μg/ml of protein A in sodium bicarbonate buffer, pH 9.5; 100μl per tube) were incubated with antiserum IgG479 (1mg/ml in PBS; 100μl per tube) for 4 hours at 4°C. Following washing with PBS and blocking with Blotto blocker, UVB-irradiated (0.8KJ/m^2) and unirradiated, *Bam*HI-digested genomic DNA isolated from HaCat cells in PBS containing 0.5M NaCl was added to the tubes (50ng). An extra control tube including UVB-irradiated, *Bam*HI-digested genomic DNA without the addition of antiserum was also included and PCR amplified in a similar manner as the above samples. PCR amplification of genomic DNA samples was performed using HotStartTaq DNA polymerase in the same way described earlier (Section 2.11). Serially diluted *Bam*HI digests of unirradiated genomic DNA of known concentrations (0-5ng), which were PCR amplified alongside the test DNA samples and run in parallel, were used as standards in the quantification of PCR products. PCR digests were analysed on a 2.0% agarose gel and quantified with QuantiScan BIOSOFT^R software. Statistical analyses were performed using GraphPad Prism, version 2.01 (Section 2.12).

6.3.4 Measurement of UVB-induced DNA damage and repair in the global genome of HaCaT cells by competitive ELISA

HaCaT cells were irradiated with increasing doses of UVB radiation (0-1KJ/m^2) as previously described (Section 2.17) or left for a period of repair (0-24 hours). Genomic DNA samples isolated from HaCaT cells were analysed for the presence of thymine dimers

by competitive ELISA in a manner similar to that described in the general protocol (Section 2.7). Single-stranded, UVC-irradiated (0.882KJ/m^2) calf thymus DNA was used as the solid phase antigen at 50μg/ml in PBS (50μl/well). Serially diluted solutions of UVB-irradiated (0-1KJ/m^2) genomic DNA (25μl/well) were used as competitors along with 25μl/well of the diluted antiserum IgG479 (1:5000 in 4% w/v dried milk/PBS). Detection of bound antiserum was achieved as previously described (Section 2.7) and the resulting absorbance was read at 492nm using a plate reader (Anthos 2001). The results were expressed as percentage inhibition of antibody binding to the solid phase antigen by competitive inhibition and statistical analysis was performed by GraphPad Software Prism, version 2.01, and Microsoft Excel 2000.

6.3.5 Measurement of thymine dimers in the cell culture medium of HaCaT cells

Measurement of thymine dimers in UVB-irradiated HaCaT cell supernatants following a period of repair (0-24 hours) was performed using competitive ELISA in a manner similar to that described in Section 2.7. Briefly, single-stranded, UVC-irradiated (0.882KJ/m^2), calf thymus DNA was used as the solid phase antigen and bound to a poly-L-lysine coated ELISA plate. Following blocking of free sites with 4% w/v dried milk/PBS (200μl/well) an aliquot of cell culture supernatants (25μl/well) from UVB-irradiated (0-1KJ/m^2) HaCat cells were applied to the plate, along with the IgG479 (1:5000 in 4% w/v dried milk/PBS; 25μl/well). Detection of primary antiserum was achieved as previously described and the resulting absorbance was read at 492nm using Anthos 2001 plate reader. The results were expressed as percentage inhibition and analysed by GraphPad Software Prism, version 2.01, and Microsoft Excel 2000 (Section 2.7).

6.3.6 Quantification of UVB-induced T<>T in the global genome of HaCaT cells by competitive ELISA

Competitive ELISA was performed in a manner similar to that described in Section 2.7 using an established concentration of genomic DNA isolated from UVB-irradiated HaCaT cells. Single-stranded, UVC-irradiated, calf thymus DNA (0.882KJ/m^2) was used as the solid phase antigen and solutions of UVB-irradiated (0-1KJ/m^2), serially diluted genomic DNA (6.25μg/ml in 4% w/v dried skimmed milk/PBS) were used as competitors (25μl/well), along with the IgG479 (1:5000 in 4% w/v dried milk/PBS; 25μl/well). Detection of primary antiserum was achieved as previously described and the resulting absorbance was read at 492nm using Anthos 2001 plate reader (Section 2.7). The results were expressed as percentage inhibition and analysed by GraphPad Software Prism, version 2.01, and Microsoft Excel 2000.

6.4 RESULTS

6.4.1 Establishment of the specificity of polyclonal antiserum IgG479 to recognise UVB-induced DNA damage in the human H-*ras* gene by ICPCR assay

The sensitivity and specificity of antiserum IgG479 to recognise DNA damage induced by UVB radiation in gene-specific sequences within the human H-*ras* gene was investigated using ICPCR assay. Following immunocapture of UVB-irradiated (0.8KJ/m^2), *Bam*HI-digested genomic DNA by the antiserum IgG479, PCR amplified DNA digests were analysed by agarose gel electrophoresis. Results showed specific amplification of the 149bp target region of the H-*ras* gene (Figure 6.1; lane 1). Non-specific binding of antiserum to control, unirradiated DNA was not detected (Figure 6.1; lane 2). In addition, although UVB-irradiated (0.8KJ/m^2) genomic DNA was used in ICPCR, the PCR product of interest was not detected (Figure 6.1; lane 3) in the absence of the antibody from the tube, confirming again the need of the antibody in ICPCR assay in order to achieve specific immunocapture of DNA damage in gene-specific sequences following UVB radiation.

Serially diluted *Bam*HI-digests of unirradiated genomic DNA of known concentration (0-5ng) were used as standards in the quantification of PCR products by comparing the band intensity of the 149bp PCR product. The intensity of the PCR band was stronger when a higher amount (5ng) of template DNA (unirradiated, *Bam*HI-digested genomic DNA; Figure 6.1; lane 5) was used in ICPCR compared with those with a lower amount of template DNA (Figure 6.1; lanes 4, 6-7). However, the intensity of the PCR band increased with increasing the amount of template DNA (50ng of UVB-irradiated, *Bam*HI-digested

genomic DNA; lane 1) in ICPCR, suggesting that the amount of the PCR product is proportional to the initial amount of template DNA.

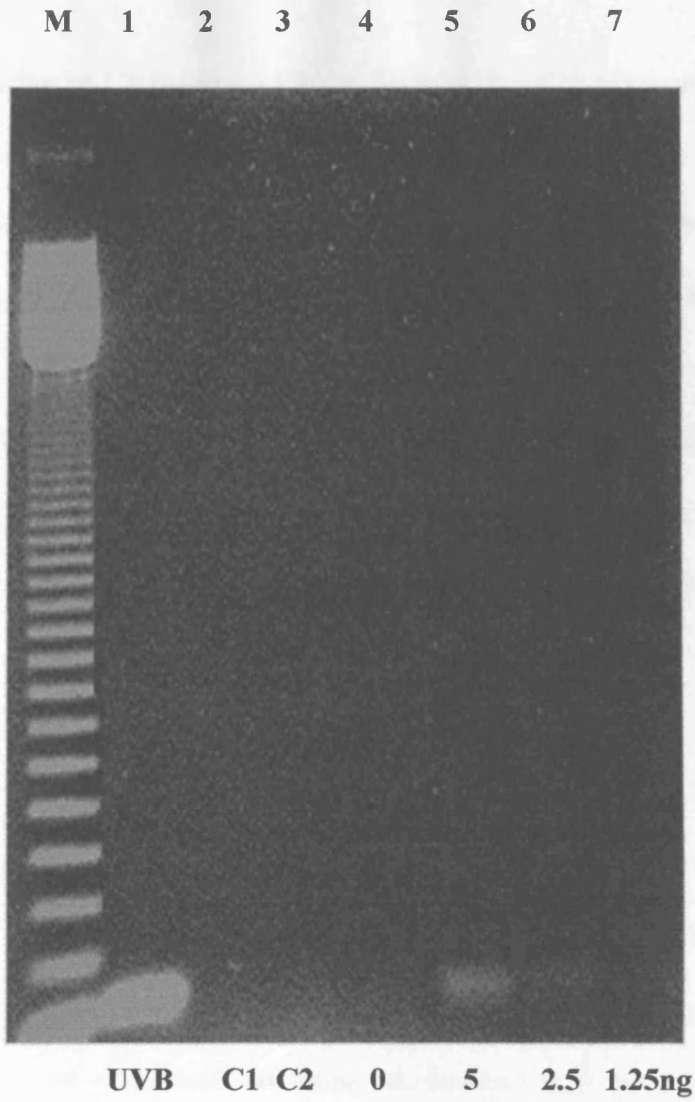


Figure 6.1: Detection of UVB-induced DNA damage in the human H-*ras* gene by polyclonal antiserum IgG479. Genomic DNA samples, isolated from UVB-irradiated (0.8KJ/m^2) HaCaT cells, were digested with *Bam*HI restriction endonuclease and immunocaptured by antiserum IgG479. PCR amplified digests (50ng) of genomic DNA were assayed by ICPCR. Lanes 1 shows 50ng of UVB-irradiated genomic DNA (0.8KJ/m^2). Lanes 2-3 show control, C1-unirradiated and C2-UVB-irradiated, *Bam*HI-digested genomic DNA (0.8KJ/m^2/no antibody) respectively. Lanes 4-7 show PCR standards (0-5ng unirradiated, *Bam*HI-digested genomic DNA) run in parallel. 40µl PCR aliquots were used. M, 123bp DNA Ladder.

6.4.2 Quantification of UVB-induced T<>T in the human H-*ras* gene

Quantification of DNA damage within gene-specific sequences in the human H-*ras* gene was performed by ICPCR assay in a manner similar to that described by Denissenko *et al.* (1996). Genomic DNA, isolated from HaCaT cells following exposure to different doses of UVB radiation (0-1KJ/m^2), was digested with *Bam*HI restriction endonuclease and immunocaptured by antiserum IgG479. Different amounts (0-100ng) of the immunorecovered template DNA were PCR amplified using gene-specific H-*ras* 7 and 8 primers and PCR products were analysed by agarose gel electrophoresis.

PCR amplification of UVB-irradiated (0.8KJ/m^2) DNA digests (10 –100ng) showed a highly significant ($p<0.0001$), linear relationship between the initial amount of template DNA and the resulting amount of PCR product ($r^2=0.99$; Figure 6.2), as the higher the amount of PCR amplified DNA the stronger the intensity of the PCR signal. This linear relationship verified that the ICPCR values were obtained by measurements of the template concentration of T<>T dimer and not from interferences with the action of HotStartTaq DNA polymerase or *Bam*HI restriction endonuclease.

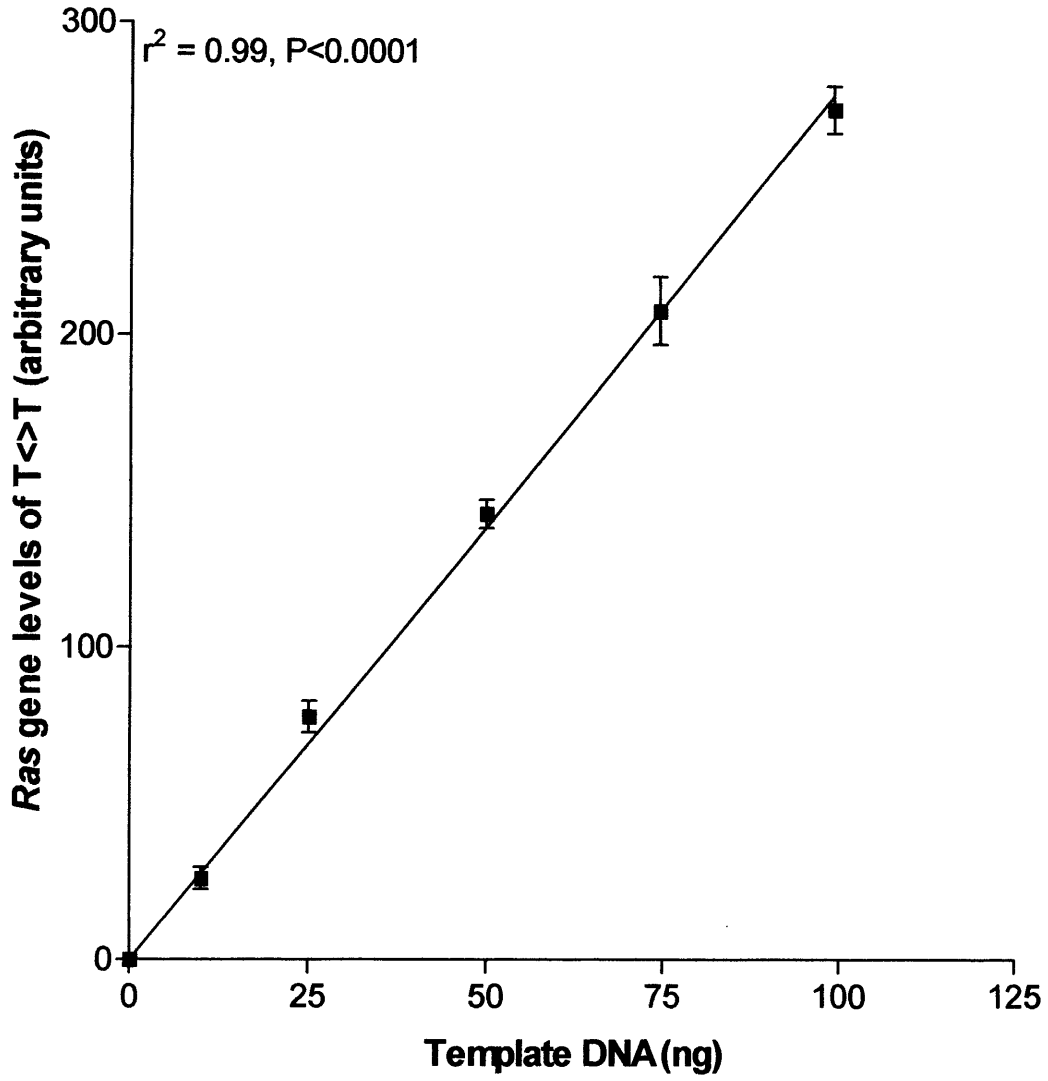


Figure 6.2: Quantification of UVB-induced T<>T in gene-specific sequences within the human H-*ras* gene. Different amounts (0-100ng) of UVB irradiated (0.8KJ/m^2), *Bam*HI-digested genomic DNA, isolated from HaCaT cells, were assayed by ICPCR using IgG479 antibody. The intensity of each individual band was detected by densitometry and plotted against increasing amount of template DNA by first order linear regression (Sigmaplot). Values represent the mean (±S.D.) of three experiments per concentration.

Analysis of PCR amplification of genomic DNA, isolated from HaCaT cells following exposure to increasing doses of UVB radiation (0-1KJ/m^2), showed specific amplification of the 149bp fragment of the human H-*ras* gene (Figure 6.3; lanes 3-7). Although a constant amount (50ng) of PCR amplified genomic DNA was used in each PCR, a dose-dependent increase in antibody binding was detected (Figure 6.4), suggesting that the amount of PCR product is proportional to the initial amount of damage, as the higher the dose of UVB radiation the stronger the intensity of the PCR signal (Figure 6.3). No PCR product was detected in the lane containing control, unirradiated DNA fragment (Figure 6.3; lane 2), indicating the specificity of the ICPCR assay to detect UVB-induced DNA damage *in vivo*. Moreover, the presence of the PCR band in the lane containing UVB-irradiated (0.8KJ/m^2), *Bam*HI-digested genomic DNA, which was PCR amplified alongside the test DNA samples and run in parallel in order to test any possible effects of UVB radiation on PCR, showed UVB radiation to have no effect on the reaction making it thus, suitable for the treatment of HaCat cells and subsequent quantification of UVB-induced DNA damage by ICPCR.

However, the response of the quantification of UVB-induced T<>T by ICPCR started to plateau at UVB doses above 0.8KJ/m^2 for 50ng of PCR amplified genomic DNA. By decreasing the concentration of template DNA to 25ng in ICPCR experiments, a linear dose-dependent increase in the induction of UVB-induced T<>T in gene-specific sequences within the human H-*ras* gene was detected (Figure 6.5). Thus, it can be concluded that the plateau level in ICPCR assay was caused by limitations in antibody binding given that the antibody had bound to all available sites for the lesions in the tube and not from a decrease in the induction of T<>T at high doses of UVB radiation. This observation of a plateau level at high doses of UVB radiation, agreed with previous results

obtained from the immunocapture of genomic DNA isolated from human fibroblast cells after *in vivo* treatment with *anti*-BPDE genotoxin by polyclonal antibody BP1, where a plateau level in immunocapture assay was detected at *anti*-BPDE concentrations above 0.5μM for 25ng of genomic DNA (Denissenko *et al.*, 1994), which was overcome by decreasing the amount of DNA in the assay.

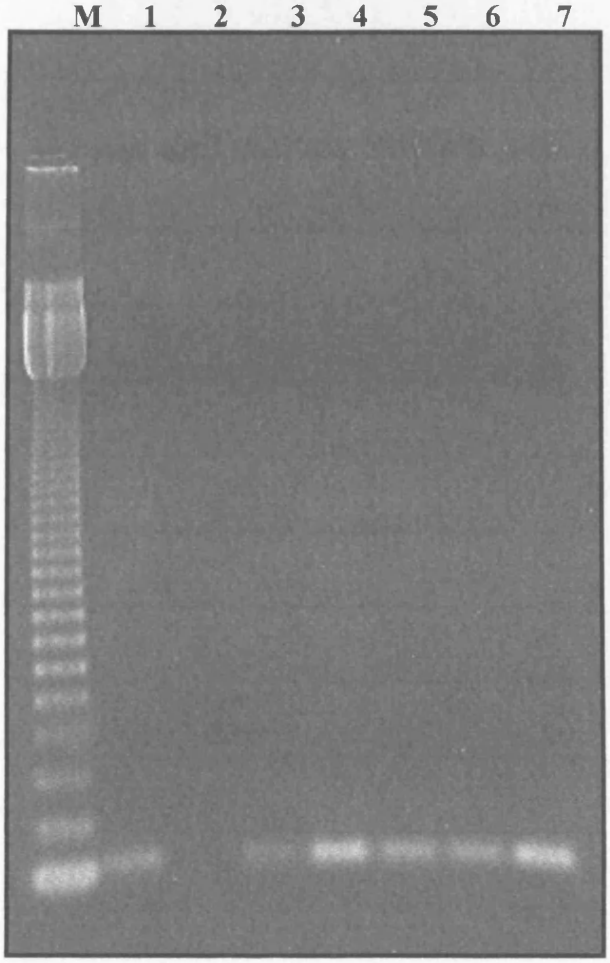


Figure 6.3: ICPCR quantification of UVB-induced DNA damage in the human H-*ras* gene *in vivo*. HaCaT cells were exposed to increasing doses of UVB radiation (0 to 1KJ/m^2). Genomic DNA, isolated from cells, was digested with *Bam*HI restriction endonuclease and 50ng digests were assayed by ICPCR using polyclonal antiserum IgG479. PCR products were analysed on a 2% agarose gel. Lanes 1-2 show control, UVB-irradiated (0.8KJ/m^2) and unirradiated, *Bam*HI-digested genomic DNA respectively. Lanes 3-7 show UVB-irradiated, *Bam*HI-digested genomic DNA. 40µl PCR aliquots were used. M, 123bp DNA Ladder.

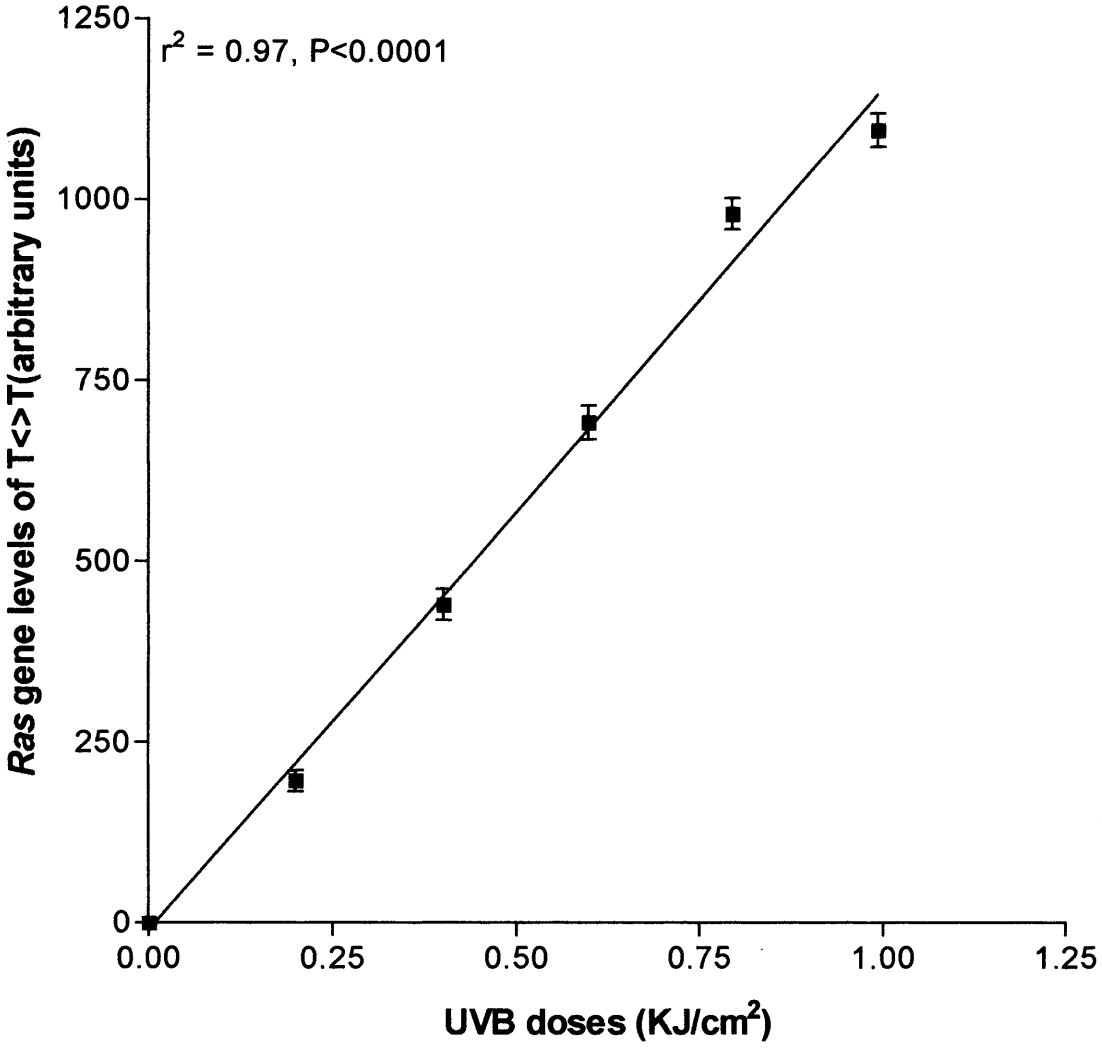


Figure 6.4: Quantification of UVB-induced T<>T in the human H-*ras* gene by ICPCR. Genomic DNA, isolated from UVB-irradiated (0 to 1KJ/m^2) HaCaT cells, was digested with *Bam*HI enzyme and 50ng digests were assayed by ICPCR using polyclonal antiserum IgG479. The relative peak intensities were determined for each band and plotted against corresponding doses of UVB radiation by first order linear regression (Sigmaplot). Data represents the mean (±S.D.) of three experiments per UVB dose.

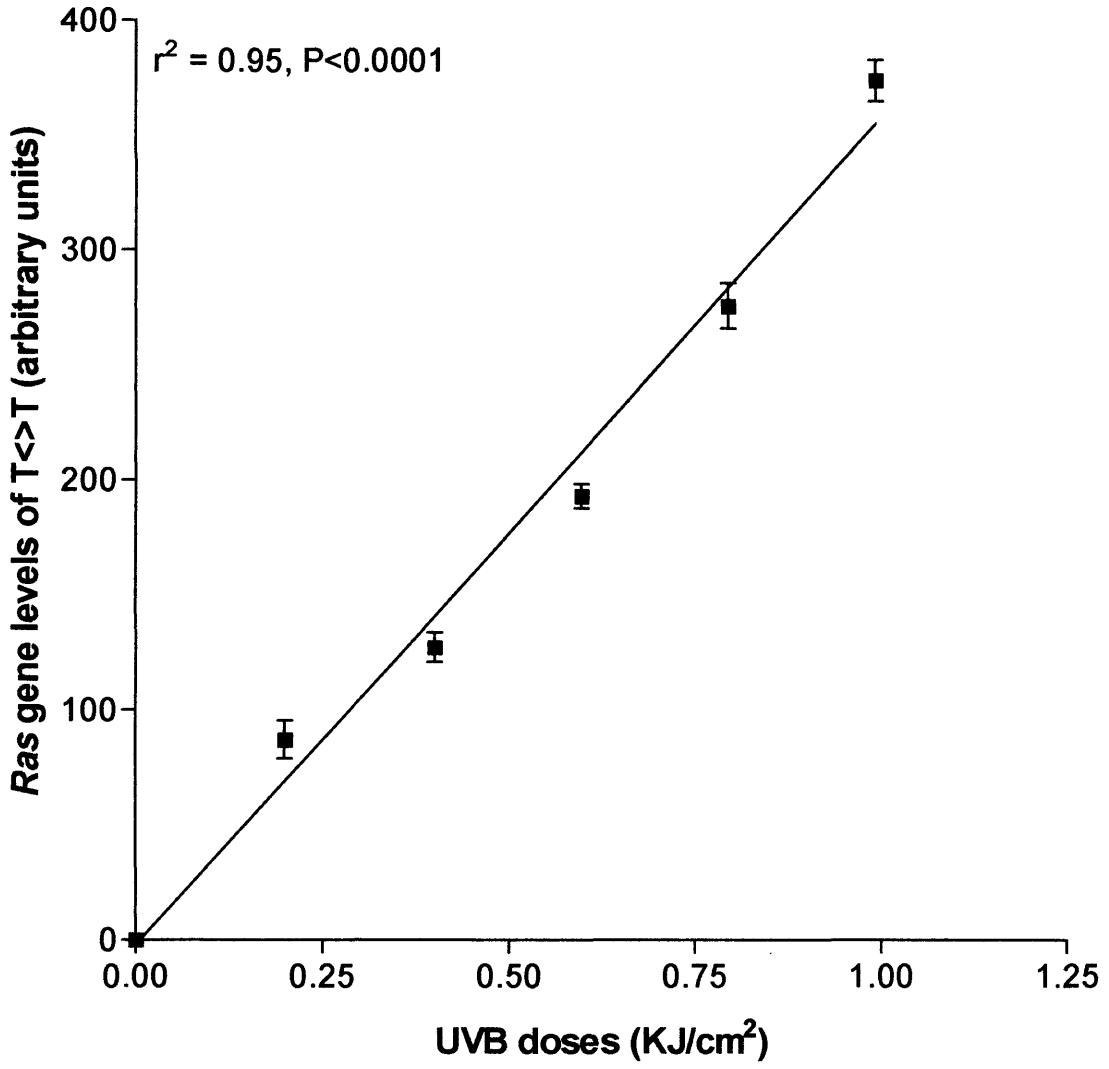


Figure 6.5: ICPCR quantification of UVB-induced T<>T in human H-*ras* gene. Genomic DNA, isolated from UVB-irradiated (0 to 1KJ/m^2) HaCaT cells, was digested with *Bam*HI enzyme and 25ng digests were quantified by ICPCR using polyclonal antiserum IgG479. The relative peak intensities were determined for each band and plotted against corresponding doses of UVB radiation by first order linear regression (Sigmaplot). Data represents the mean (±S.D.) of three experiments per UVB dose.

6.4.3 Global measurement of UVB-induced T<>T in HaCaT cells by competitive ELISA

Measurement of DNA damage induced by different doses of UVB radiation (0-1KJ/m^2) in the global genome of HaCaT cells was performed by competitive ELISA as described earlier. In competitive ELISA, inhibition of antiserum binding to solid-phase antigen [single-stranded, UVC-irradiated (0.882KJ/m^2) calf thymus DNA] was related to the dose of UVB radiation (Figure 6.6). The lowest UVB dose (0.2KJ/m^2) was the least effective inhibitor with an IC_{50} of 18.12μg/ml, but with increasing doses of UVB radiation, the effectiveness of inhibition increased, being most apparent between 1.0KJ/m^2 and 0.2KJ/m^2 UVB dose (Table 6.1). The IC_{50} is the concentration of antigen, giving 50% inhibition, and provides an index of the antigenicity of the compound as the lower the value the better the competitor. No competition was demonstrated for control, unirradiated genomic DNA, clearly indicating the specificity of antiserum to recognise thymine dimers in HaCaT cells following exposure to UVB radiation.

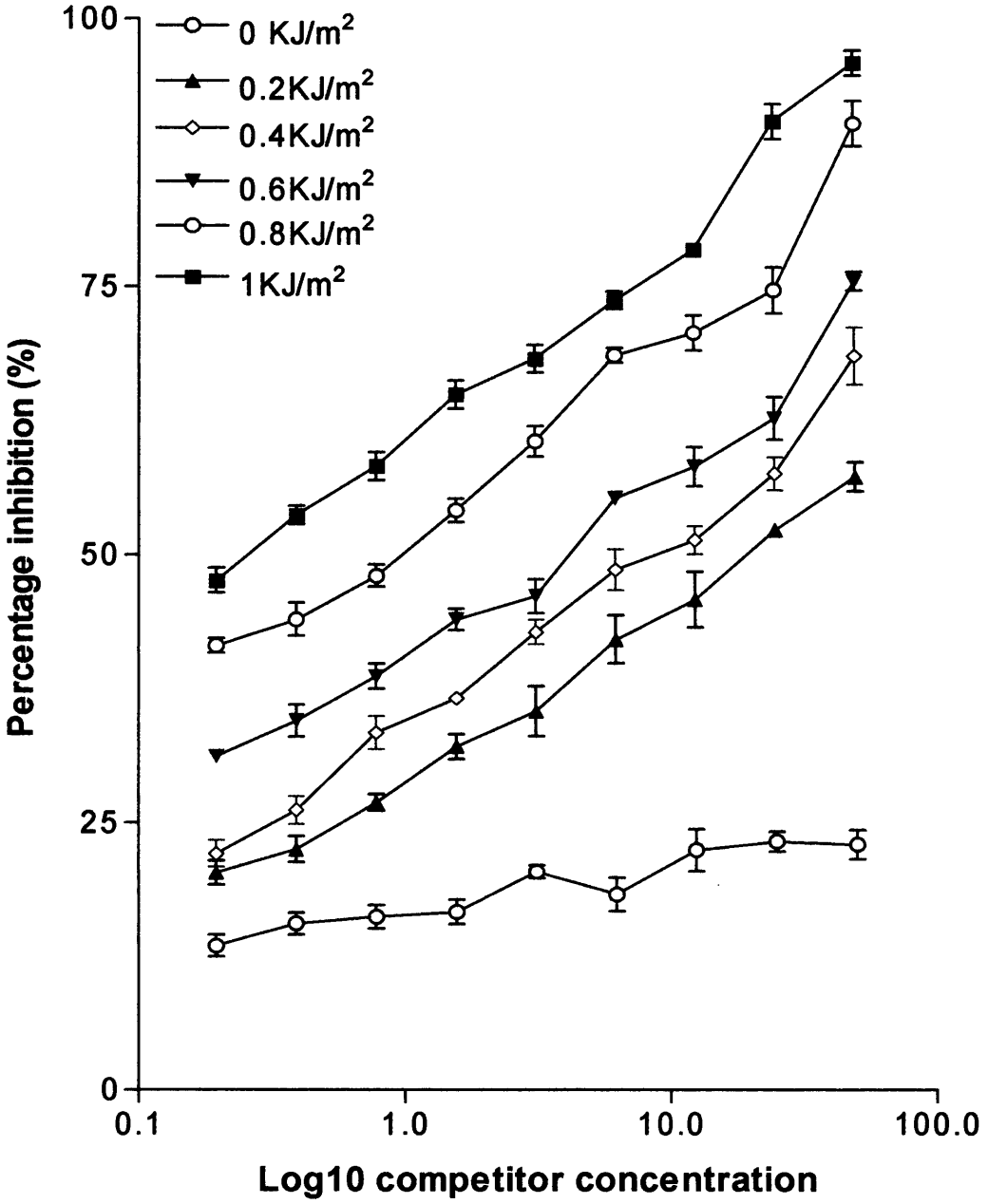


Figure 6.6: Global measurement of DNA damage in HaCaT cells following exposure to UVB radiation by competitive ELISA. HaCaT cells were exposed to increasing doses (0-1KJ/m^2) of UVB radiation. Genomic DNA samples (50μg/ml), isolated from cells, were assayed by competitive ELISA using polyclonal antiserum IgG479. Values represent the mean (±S.D.) of three determinations per concentration.

TABLE 6.1: Summary of competitive inhibition of antibody binding to UVB-irradiated genomic DNA isolated from HaCaT cells following exposure to increasing doses of UVB radiation (0-1KJ/m^2). Unirradiated genomic DNA (0KJ/m^2) did not compete (IC_{50} >50μg/ml).

Inhibitor (UVB doses in KJ/m^2)	IC_{50} (μg/ml)
1	0.24
0.8	0.96
0.6	4.14
0.4	8.45
0.2	18.12
0	>50

A concentration of 6.25μg/ml of genomic DNA isolated from UVB-irradiated HaCaT cells was found to be the best inhibitor for antiserum binding giving an IC_{50} of 0.44μg/ml (Figure 6.7) compared to higher or lower concentrations of genomic DNA, which showed less inhibition or little inhibition with the antiserum IgG479 (IC_{50}>50 μg/ml). Thus, 6.25μg/ml of UVB-irradiated genomic DNA was used in the comparison of levels of T<>T dimers between gene-specific sequences within the human H-*ras* gene and the global genome of HaCaT cells after exposure to UVB radiation.

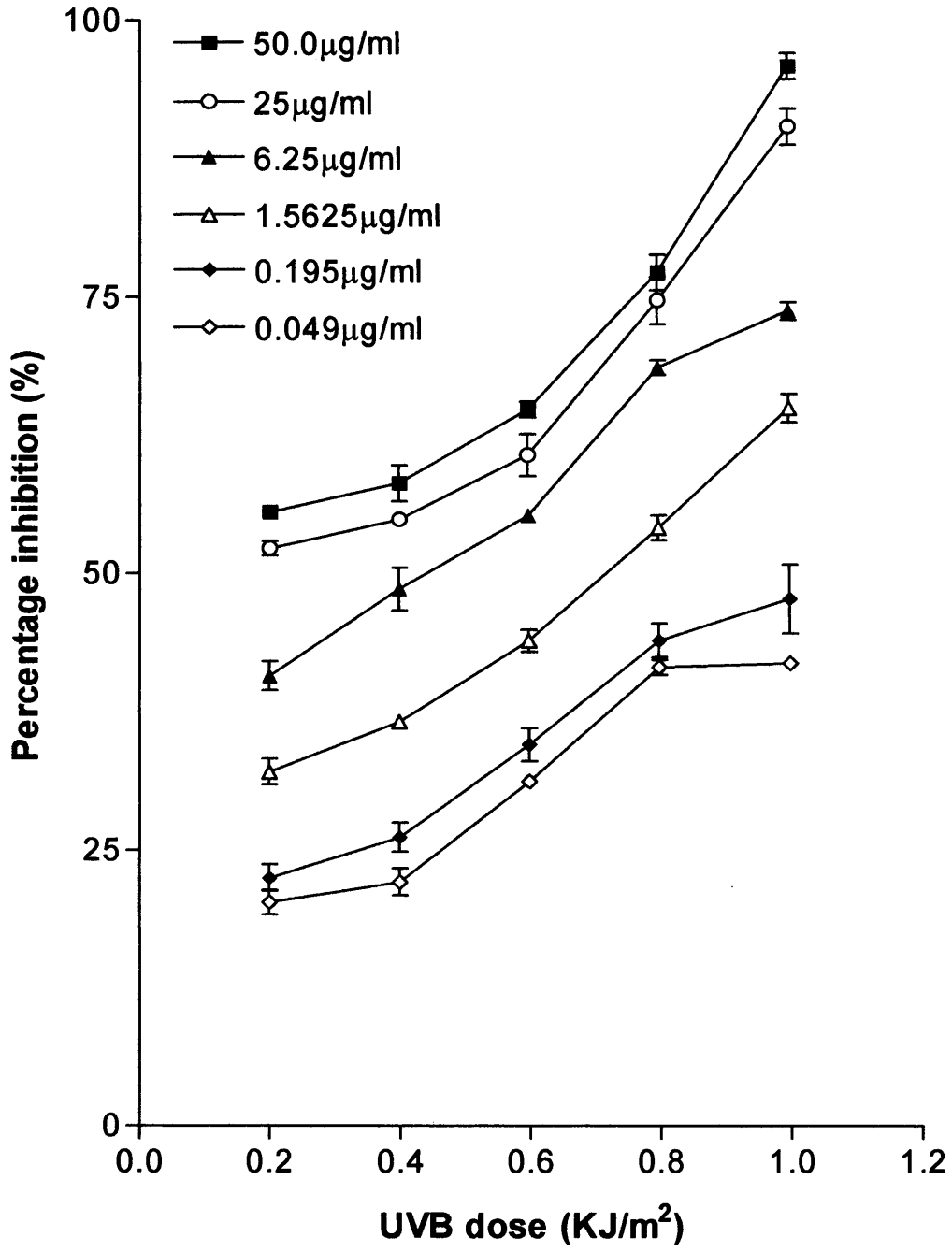


Figure 6.7: Global measurement of UVB-induced T<>T in HaCat cells by competitive ELISA. Genomic DNA samples (50µg/ml), isolated from HaCat cells after exposure to increasing doses (0-1KJ/m^2) of UVB radiation, were assayed by competitive ELISA using polyclonal antiserum IgG479. Values represent the mean (±S.D.) of three determinations per concentration.

6.4.4 Comparison of gene- and global measurements of T<>T in HaCat cells

Genomic DNA, isolated from HaCaT cells following exposure to UVB radiation (0-1KJ/m^2), was assayed either by ICPCR or competitive ELISA for the quantification of UVB-induced T<>T in gene-specific sequences within the human H-*ras* gene and the global genome respectively. Comparison of the results obtained from ICPCR assay with those obtained by competitive ELISA showed a significant correlation (r^2 = 0.84, P<0.0001) between measures of T<>T in the H-*ras* gene and the global genome in HaCaT cells (Figure 6.8).

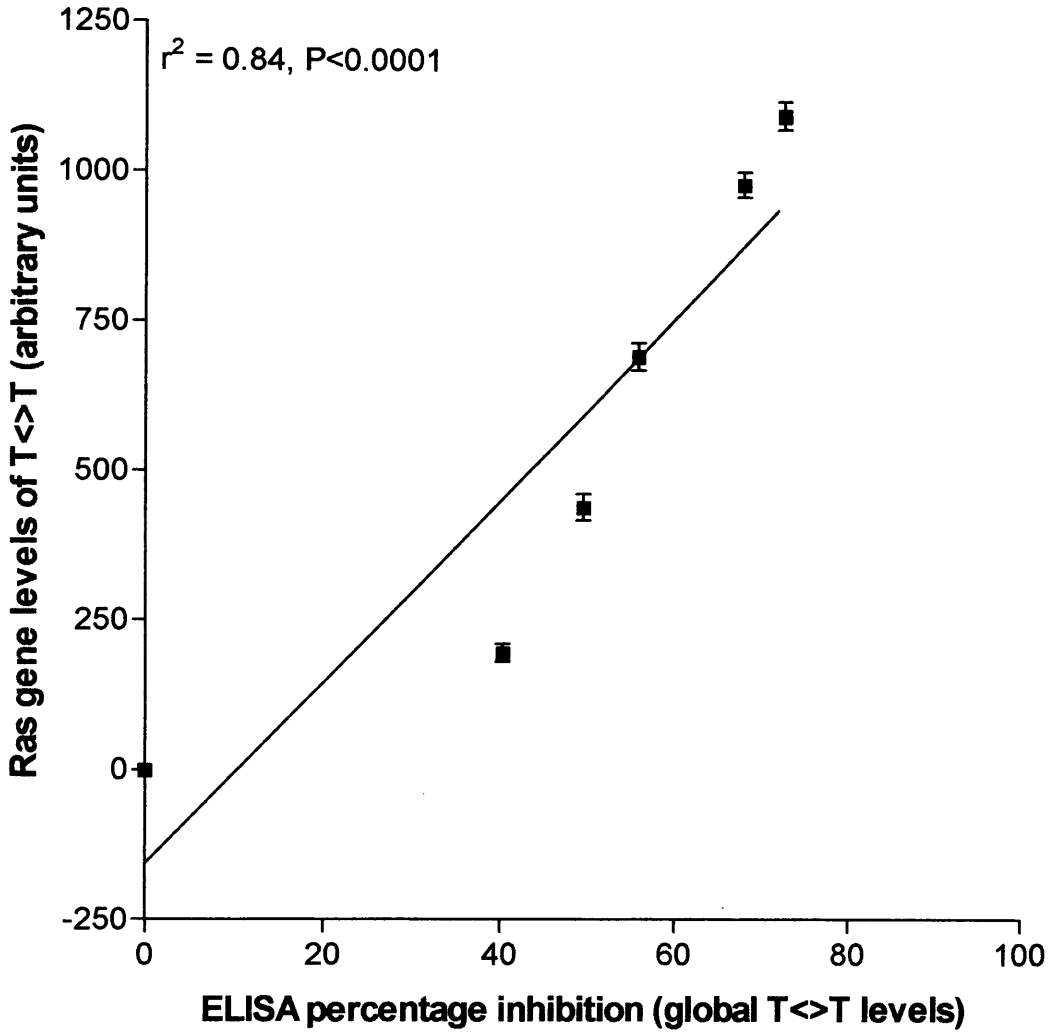


Figure 6.8: Comparison of gene and global genome measures of T<>T in UVB-irradiated HaCaT cells. Genomic DNA, isolated from HaCaT cells following exposure to increasing doses of UVB radiation (0-1KJ/m^2), was assayed by ICPCR and compared to UVB-induced T<>T levels in the global genome analysed by competitive ELISA. The levels of T<>T in gene-specific sequences within the human H-*ras* gene were plotted against those in the global genome by first order linear regression (Sigmaplot). Values represent the mean (±S.D.) of three experiments per UVB dose.

6.4.5 MTT assay

Viability of HaCaT cells following exposure to increasing doses (0-1KJ/m^2) of UVB radiation, and after a period of repair (0-24 hours) was performed by MTT assay in a manner similar to that described by Mosmann (1983). The MTT assay is a quantitative colorimetric assay for cell survival and proliferation based on the ability of cells to cleave MTT to an insoluble formazan product that is subsequently dissolved by the addition of DMSO into the cells. The assay detects only viable cells as MTT is cleaved in active mitochondria by cellular enzymes (mitochondrial dehydrogenases) associated with metabolic activity. Thus, MTT dye reduction is proportional to cell number (Mosmann, 1983).

Exposure of cells to increasing doses of UVB radiation resulted in a dose-dependent decrease in the reduction of MTT to its formazan product, as the higher the UVB dose the lower the amount of MTT cleaved by HaCaT cells over the period studied. High doses of UVB radiation (0.8 and 1KJ/m^2) resulted in an approximately 64% and 68% of MTT reduction after 6 hours post-irradiation respectively (Figure 6.9) compared to low UVB doses (0.2 and 0.4 KJ/m^2), which corresponded to 75% and 73% of MTT reduction respectively within the same period of incubation. A significant ($p<0.05$) decrease in cell viability was detected following 4 hours incubation for all UVB doses, which was followed by three further significant decreases ($p<0.001$) between 6 to 24 hour incubation compared to the control, unirradiated cells (Figure 6.9). No significant differences in the viability of cells were seen for the 1 and 2 hour time points.

UVB-irradiation of HaCaT cells with 1000J/m^2 resulted in an approximately 40% of cell survival following 24 hours after UVB-irradiation. MTT assay was reported to measure

cell proliferation (Mossman, 1983). It can be assumed, therefore, that although, a reduction in MTT dye was detected by increasing UVB doses, and due to the requirement of cell to cell contact for growth (Boukamp *et al.*, 1988), cells were still viable after 24 hours post-UVB-irradiation with 1000J/m^2 but unable to proliferate. This hypothesis agreed with the results obtained by Greinert *et al.* (2000), where the measurement of the repair kinetics of HaCaT cells after irradiation with similar UVB doses by flow cytometry, showed the cells to be viable and synchronized in the G1 phase of the cell cycle by contact inhibition without proliferating. The apparent high loss of HaCaT cell viability observed following irradiation with high UVB doses did not affect the quantification of the repair of UVB-induced DNA damage in the H-*ras* gene and the total genome of the HaCaT cells as T<>T dimers were shown to be efficiently repaired in HaCaT cells by ICPCR and ELISA assay respectively.

MTT assay provides a rapid and versatile method for assessing cell viability, proliferation and cytotoxity, compared to common methods such as trypan blue exclusion dye or incorporation of nucleotides during cell proliferation (5-bromo-2'deoxyuridine or ^{3}H-thymidine), which though sensitive, are usually limited by the impracticability of processing large numbers of samples or by the requirement of radioisotopes.

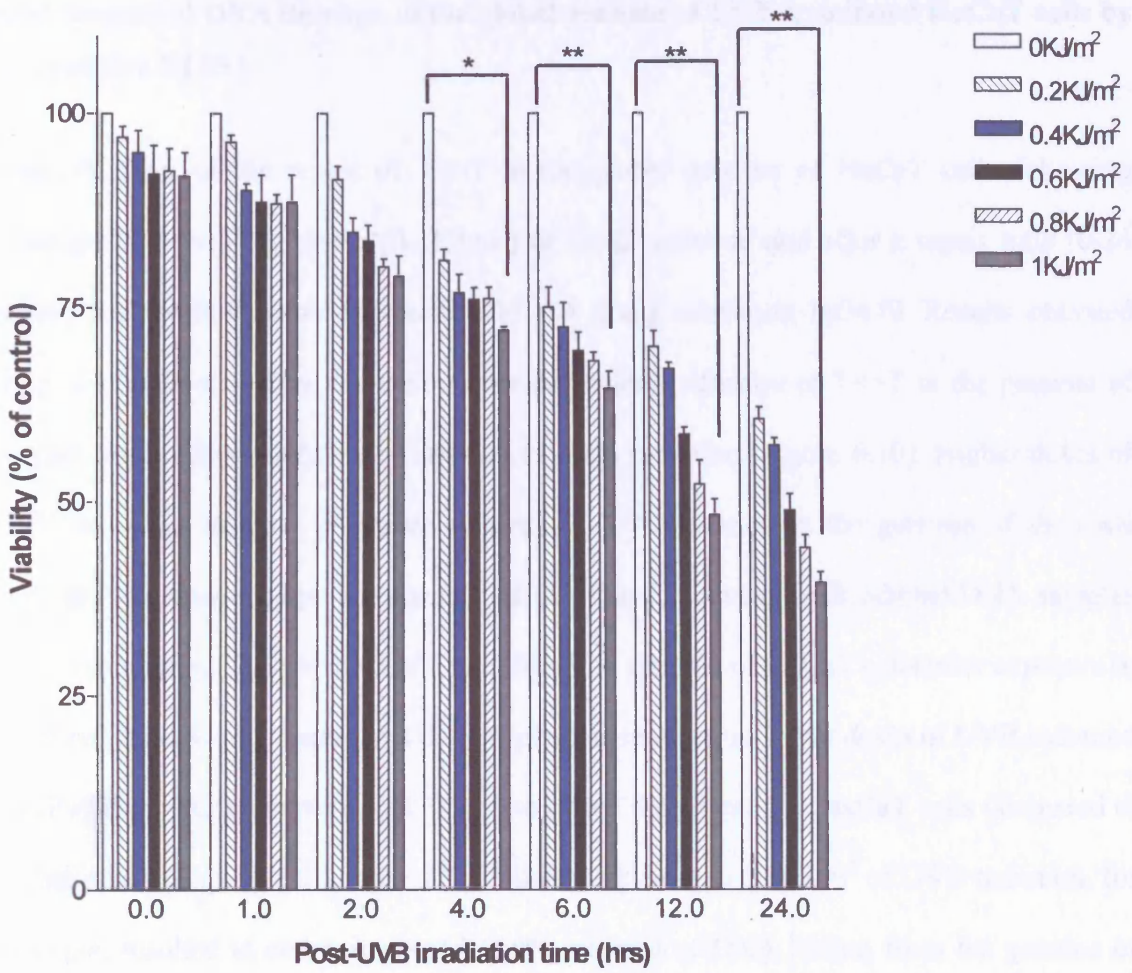


Figure 6.9: The effects of UVB radiation on the viability/proliferation of HaCaT cells. Cells were exposed to increasing doses of UVB radiation (0-1KJ/m^2) and after a repair time (0-24 hours) were assayed by MTT assay. Values represent the mean (±S.D.) of three experiments per UVB dose. Asterisks indicate a statistically significant (*$p<0.05$, **$p<0.001$) difference compared to control, untreated cells, determined by Student's *t* test.

6.4.6 Repair of DNA damage in the global genome of UVB-irradiated HaCaT cells by competitive ELISA

Quantification of the repair of T<>T in the global genome of HaCaT cells following exposure to increasing doses (0-1KJ/m^2) of UVB radiation and after a repair time (0-24 hours) was performed by competitive ELISA using antiserum IgG479. Results obtained from competitive ELISA, showed a dose-dependent induction of T<>T in the genome of HaCaT cells immediately after exposure to UVB radiation (Figure 6.10). Higher doses of UVB radiation resulted in higher amounts of T<>T formed in the genome of the cells compared to lower doses. The amount of T<>T in the control, unirradiated DNA samples was insignificant. The removal of T<>T from the genome of HaCaT cells after exposure to UVB radiation was occurred in a dose-dependent manner, as lower doses of UVB radiation resulted in higher amounts of T<>T excised from the genome of HaCaT cells compared to higher doses (Figures 6.10 and 6.11). Cellular exposure to 0.2KJ/m^2 of UVB radiation, for example, resulted in an approximately 69% removal of DNA lesions from the genome of the cells after 6 hours of repair compared to the highest UVB dose (1KJ/m^2), where 48% of lesions were still present within the same period. Furthermore, though dose-dependent, the removal of T<>T levels from the HaCaT genome showed a time-dependence too, as longer periods of repair required for excision of T<>T from the genome by increasing doses of UVB radiation. A significant increase ($p<0.05$) in T<>T removal was detected following 2 hours of repair, which was followed by two further significant increases ($p<0.001$) for the 4 and 6 hour time points compared to the control, unirradiated samples (Figure 6.10). Maximal increase in the removal of the lesions from the HaCaT genome, over the period studied, was detected after 12 and 24 hour post-irradiation ($p<0.0001$), where T<>T levels returned almost to control, unirradiated levels (Figure 6.10). On the whole genome of HaCaT cells, about 21%, 23%, 25%, 26% and 28% of T<>T corresponding to increasing

doses of UVB radiation (0.2-1KJ/m^2) had still not been repaired within 24 hours after irradiation.

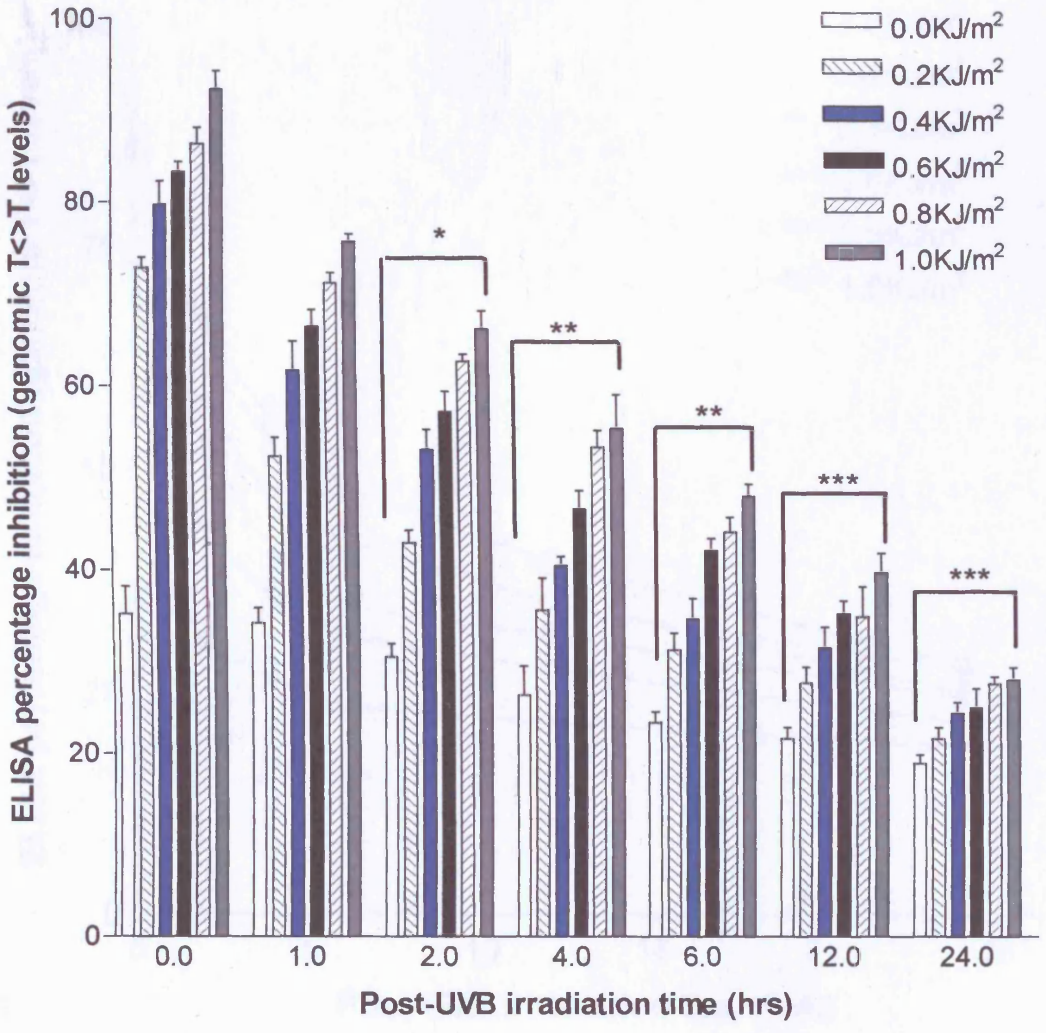


Figure 6.10: Measurement of thymine dimers in the genome of HaCaT cells following exposure to UVB radiation. HaCaT cells were exposed to increasing doses (0-1KJ/m^2) of UVB radiation and genomic DNA, isolated from cells at post-treatment times (0-24 hours), was assayed by competitive ELISA. Levels of thymine dimers formed in the genome of HaCaT cells were expressed as percentage inhibition with antiserum IgG479 and plotted against corresponding repair times. Values represent the mean (±S.D.) of three experiments per UVB dose. Asterisks indicate a statistically significant (*$p<0.05$, **$p<0.001$ and ***$p<0.0001$) difference compared to control, unirradiated cells, determined by Student's *t* test.

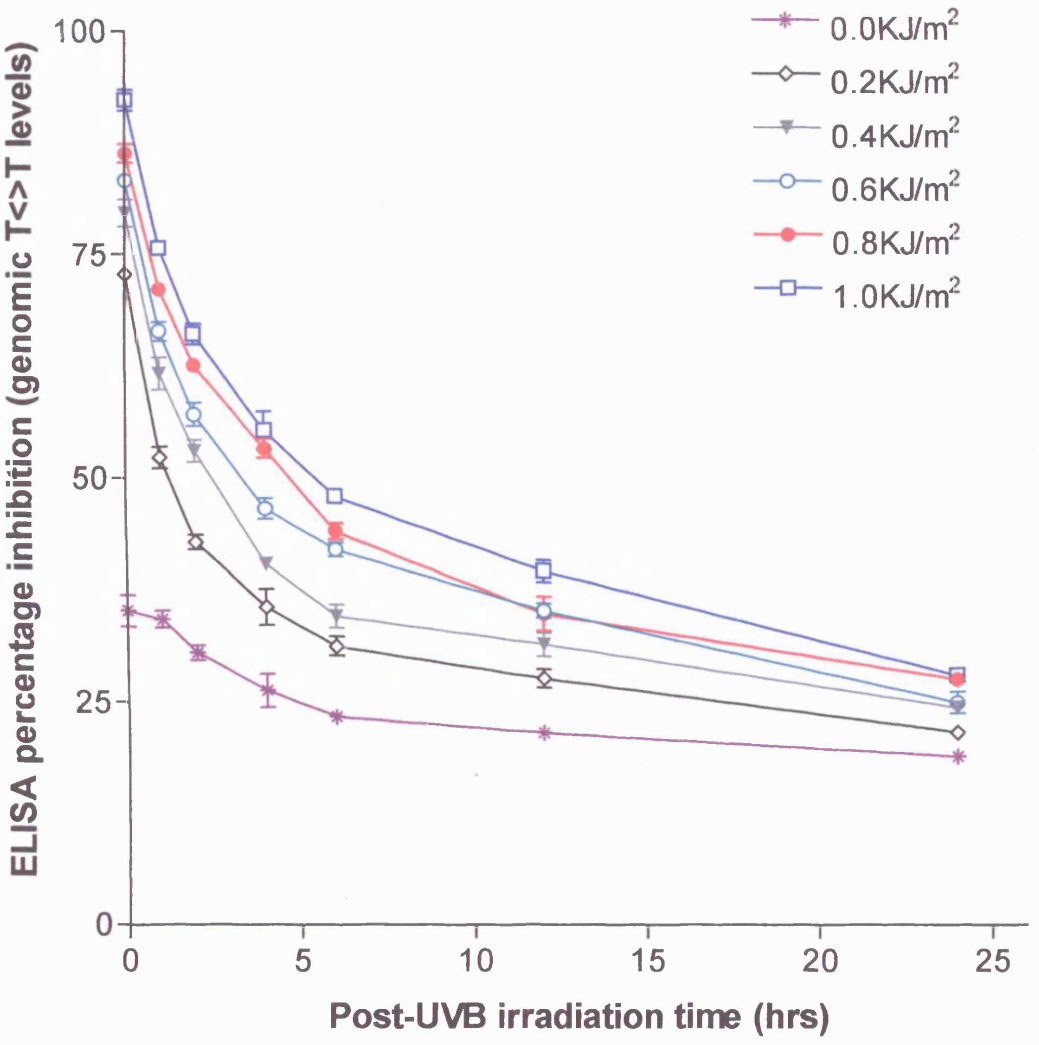


Figure 6.11: Measurement of UVB-induced thymine dimers in the genome of HaCaT cells. Genomic DNA samples, isolated from UVB-irradiated (0-1KJ/m^2) HaCaT cells at post-treatment times (0-24 hours), were analysed by competitive ELISA. Levels of thymine dimers formed in the genome of HaCaT cells were expressed as percentage inhibition with antiserum IgG479. Values represent the mean (±S.D.) of three experiments per UVB dose.

6.4.7 Measurement of thymine dimers in the supernatants of HaCaT cells

Measurement of UVB-induced T<>T in the supernatants of HaCaT cells following irradiation of cells with UVB (0-1KJ/m^2) and after a repair period (0-24 hours), was performed using competitive ELISA. The removal of T<>T from HaCaT cells occurred in a dose-dependent manner following incubation at post-treatment times. Exposure of cells to 0.8 and 1KJ/m^2 of UVB radiation, for example, resulted in 64% and 59% of T<>T excretion in the supernatants of HaCaT cells within 6 hours of repair respectively, compared to 83% and 75% of lesion removal following exposure to 0.2 and 0.4KJ/m^2 respectively (Figure 6.12). No changes were detected in the control, unirradiated samples. The excretion of T<>T in the supernatants of HaCaT cells followed a time-dependent increase. A significant increase ($p<0.001$) in T<>T repair was detected for 2 and 4 hours of incubation. Maximal increase in the excision of lesions from the HaCaT genome was detected at 6, 12 and 24 hour time points ($p<0.0001$) compared to control, unirradiated levels (Figure 6.12). By 24 hours of incubation, about 91%, 89%, 86%, 82% and 79% of T<>T had been excised in the supernatants of cells as repaired products following treatment with increasing doses (0.2-1KJ/m^2) of UVB radiation respectively.

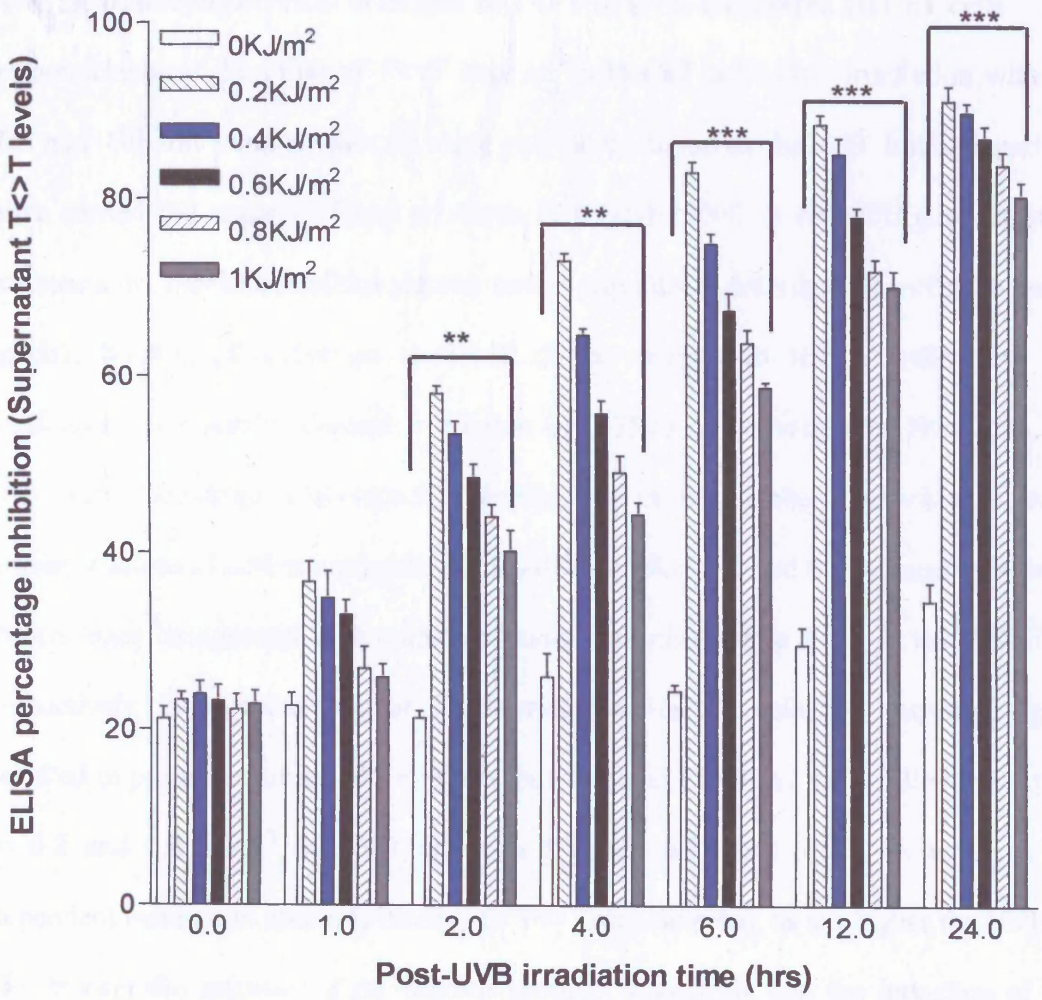


Figure 6.12: Measurement of excreted T<>T into the supernatants of UVB-irradiated HaCaT cells. HaCaT cells were exposed to increasing doses (0-1KJ/m^2) of UVB radiation and supernatants, collected at post-treatment times (0-24 hours), were assayed by competitive ELISA. Levels of T<>T excreted into the supernatants of HaCaT cells after repair were expressed as percentage inhibition with antiserum IgG479 and plotted against corresponding repair times. Values represent the mean (±S.D.) of three experiments per UVB dose. Asterisks indicate a statistically significant (**$p<0.001$ and ***$p<0.0001$) difference compared to control, unirradiated cells, determined by Student's *t* test.

6.4.8 Immunocytochemical detection of T<>T in UVB-irradiated HaCaT cells

Immunochemical detection of T<>T induced in HaCaT cells after irradiation with UVB (0.2 and 0.8KJ/m^2) was performed using polyclonal antiserum IgG479. Initial experiments were carried out using different dilutions (1:1000-1:10000 in NGS/PBS) of polyclonal antiserum in the immunofluorescence assay previously described in order to achieve specific binding of antiserum to T<>T dimers present in HaCaT cells post UVB-irradiation. The results showed a dilution of 1:7500 of antiserum in NGS/PBS to be effective in blocking any non-specific binding sites in the chamber glass whereas lower or higher dilutions of antiserum resulted in insufficient blocking and the presence of increased fluorescence background or less intense binding of antiserum to T<>T in the HaCaT cells respectively. Immunostaining of UVB-irradiated HaCaT cells by antiserum IgG479 resulted in positive staining of T<>T within the nuclei of HaCaT cells following exposure to 0.2 and 0.8 KJ/m^2 of UVB radiation (Figures 6.13 and 6.14). However, a dose-dependent increase in antibody binding to T<>T was detected, as the higher the UVB dose the stronger the intensity of the nuclear staining, suggesting that the induction of T<>T dimers in HaCaT cells is proportional to the initial dose of UVB radiation. Non-specific binding of antiserum to control, unirradiated cells was not detected, clearly indicating the specificity of polyclonal antiserum IgG479 to recognise UVB-induced T<>T *in vivo* (Figure 6.15).

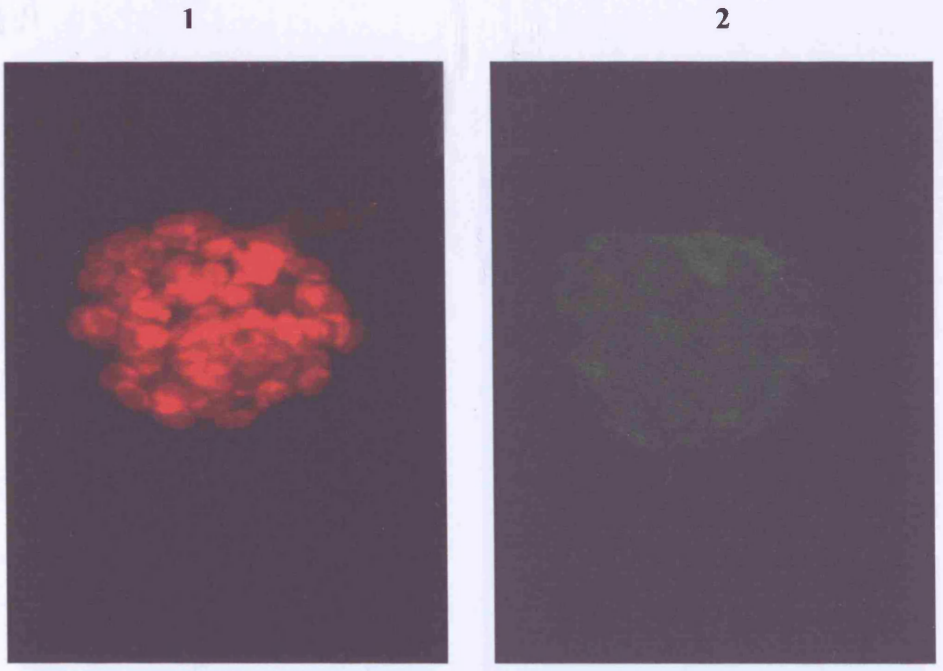


Figure 6.13: Detection of UVB-induced T<>T in HaCaT cells by indirect immunofluorescence assay. HaCaT cells were exposed to 0.8KJ/m^2 of UVB radiation and assayed by indirect immunofluorescence assay utilizing polyclonal antiserum IgG479. Intense staining of T<>T within the nucleus of cells was detected at x 400 magnification. Plate 1 shows staining of HaCaT cells with DAPI while plate 2 shows nuclear staining of cells with FITC.

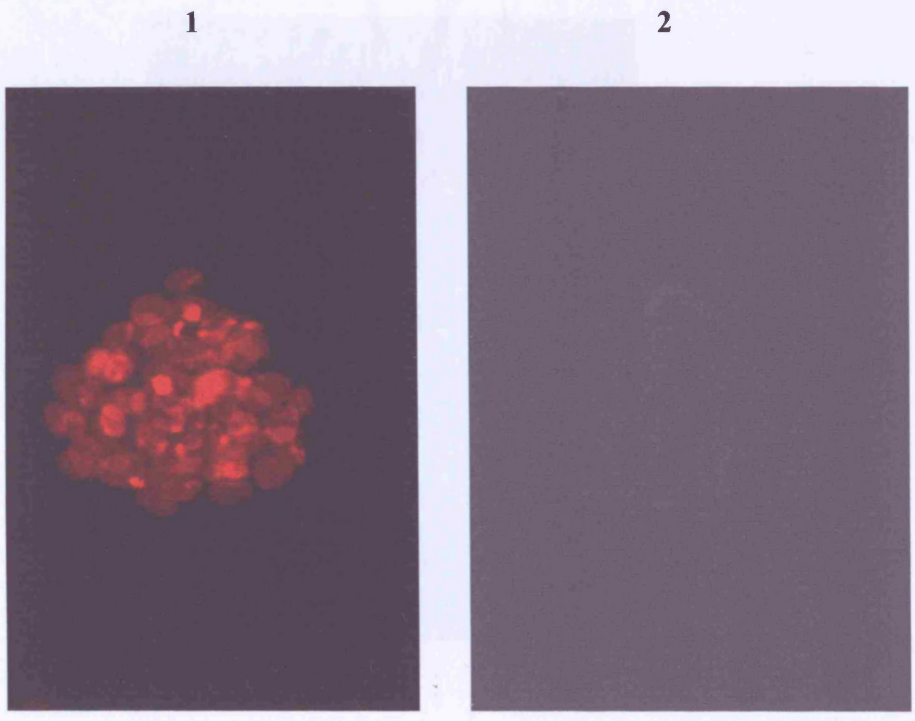


Figure 6.14: Detection of UVB-induced T<>T in HaCaT cells by indirect immunofluorescence assay. HaCaT cells were exposed to 0.2KJ/m^2 of UVB radiation and assayed by indirect immunofluorescence assay utilizing polyclonal antiserum IgG479. Intense staining of T<>T within the nucleus of cells was detected at x 400 magnification. Plate 1 shows staining of HaCaT cells with DAPI while plate 2 shows nuclear staining of cells with FITC.

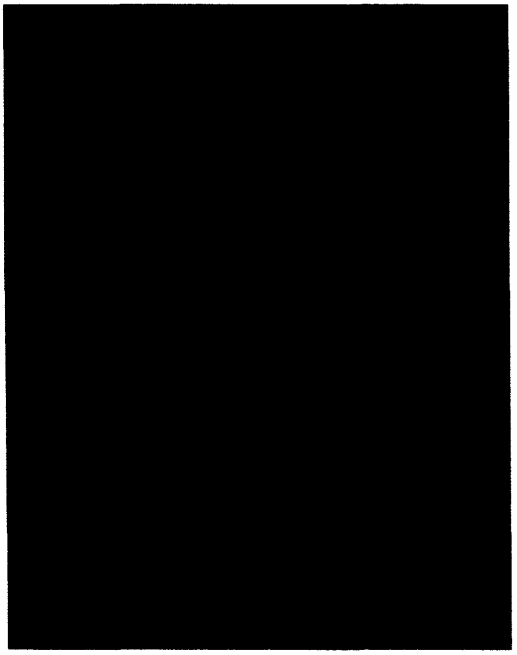


Figure 6.15: Indirect immunofluorescence assay in HaCaT cells. Unirradiated HaCaT cells were assayed by indirect immunofluorescence assay utilizing polyclonal antiserum IgG479. No staining of T<>T was detected at x 400 magnification.

6.4.9 Quantification of T<>T repair in gene-specific sequences in the human H-*ras* gene by ICPCR

Having established that immuno-coupled PCR (ICPCR) assay could be used in the assessment of UVB-induced T<>T within specific gene sequences in the human H-*ras* gene, its suitability for the quantification of the repair of these lesions was investigated. Genomic DNA, isolated from HaCaT cells following exposure to UVB radiation (0-1KJ/m^2) and after a period of repair (0 to 24 hours), was digested with *Bam*HI restriction endonuclease, immunocaptured by polyclonal antiserum IgG479 and the immuno-recovered template DNA was PCR amplified using gene-specific primers (H-*ras* 7 and 8).

PCR amplification of UVB-irradiated DNA digests following incubation at post-treatment times showed specific amplification of the 149bp fragment of the human H-*ras* gene (Figure 6.16; Gels A, B and C). A dose-dependent increase in antibody binding with increasing doses of UVB radiation was detected after a period of repair, though a constant amount of template DNA (50ng of UVB-irradiated *Bam*HI-digested genomic DNA) was used in each PCR experiment. No non-specific binding of antibody to control, unirradiated DNA was observed (Figure 6.16; Gel A; lane 2).

Repair of UVB-induced T<>T from gene-specific sequences within the human H-*ras* proto-oncogene was determined by comparing the intensity of each of the PCR bands obtained from PCR amplification of genomic DNA following exposure to increasing doses (0-1KJ/m^2) of UVB radiation and after incubation at post-treatment times (0-24 hours). Analysis of PCR amplified DNA digests by agarose gel electrophoresis showed a progressive decrease in the intensity of the PCR signal by increasing the repair time.

Longer incubation periods (Figure 6.16; Gel C; 24 hours after irradiation) of the HaCaT cells following UVB radiation resulted in a decrease in the intensity of the PCR band for the 149bp product of interest for all the UVB doses compared to that of shorter periods (Figure 6.16; Gel A and B; 4 and 6 hours after irradiation respectively) and to the 0 hour time point (immediately after irradiation; Figure 6.3).

At 1 hour after irradiation, about 95% of T<>T were still present in the cells following exposure to 1KJ/m^2 of UVB radiation compared to that of 0.2KJ/m^2, where about 40% of DNA damage had been repaired within the same period of repair (Figure 6.17). Thus, the removal of the UVB-induced T<>T from the H-*ras* gene was performed in a dose-dependent manner, as the lowest doses of UVB radiation resulted in a faster repair of DNA damage. Quantification of UVB-induced DNA repair showed a slow removal of T<>T from the H-*ras* gene up to 2 hours of repair followed by a faster repair of lesions with time (Figure 6.17). At 6 hours of repair, 80% and 54% of T<>T was repaired after irradiation with 0.2 and 1KJ/m^2 of UVB respectively compared to that of 2 hours, where for the same doses 47% and 14% of lesions was removed from the H-*ras* gene respectively. By 24 hours of repair, about 7%, 9%, 11%, 13% and 15% of T<>T were still present in the gene-specific sequences of the H-*ras* gene after exposure to increasing doses of UVB radiation (0.2-1KJ/m^2) respectively. This observation of remaining damage in the cells after a repair period agreed with the results obtained from the quantification of genomic DNA isolated from human fibroblast cells following treatment with *anti*-BPDE genotoxin, where an approximate 20% of *anti*-BPDE adducts were still present in the H-*ras* gene within 17 hours of repair (Denissenko *et al.*, 1994).

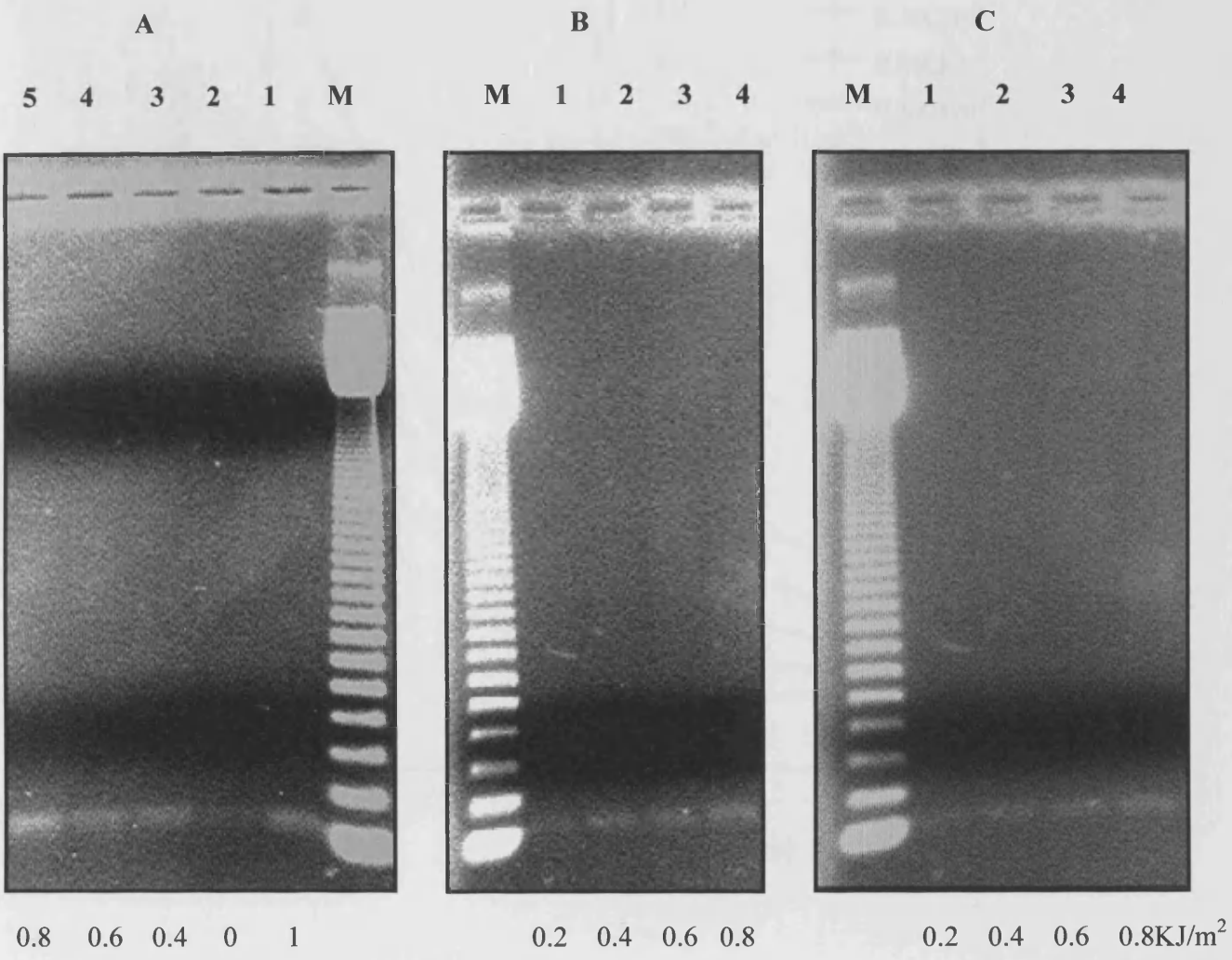


Figure 6.16: Repair of UVB-induced DNA damage in the H-*ras* gene by ICPCR. HaCaT cells were exposed to increasing doses of UVB radiation (0 to 1KJ/m^2) and genomic DNA, isolated from cells after a period of repair (0-24 hours), was *Bam*HI-digested and 50ng digests were assayed by ICPCR using polyclonal antiserum IgG479. PCR products were analysed on a 2% agarose gel. (A) Quantification of UVB-induced DNA damage after 4 hrs of repair, (B) 6 hrs of repair and (C) 24 hrs of repair. 40µl PCR aliquots were used. M, 123bp DNA Ladder.

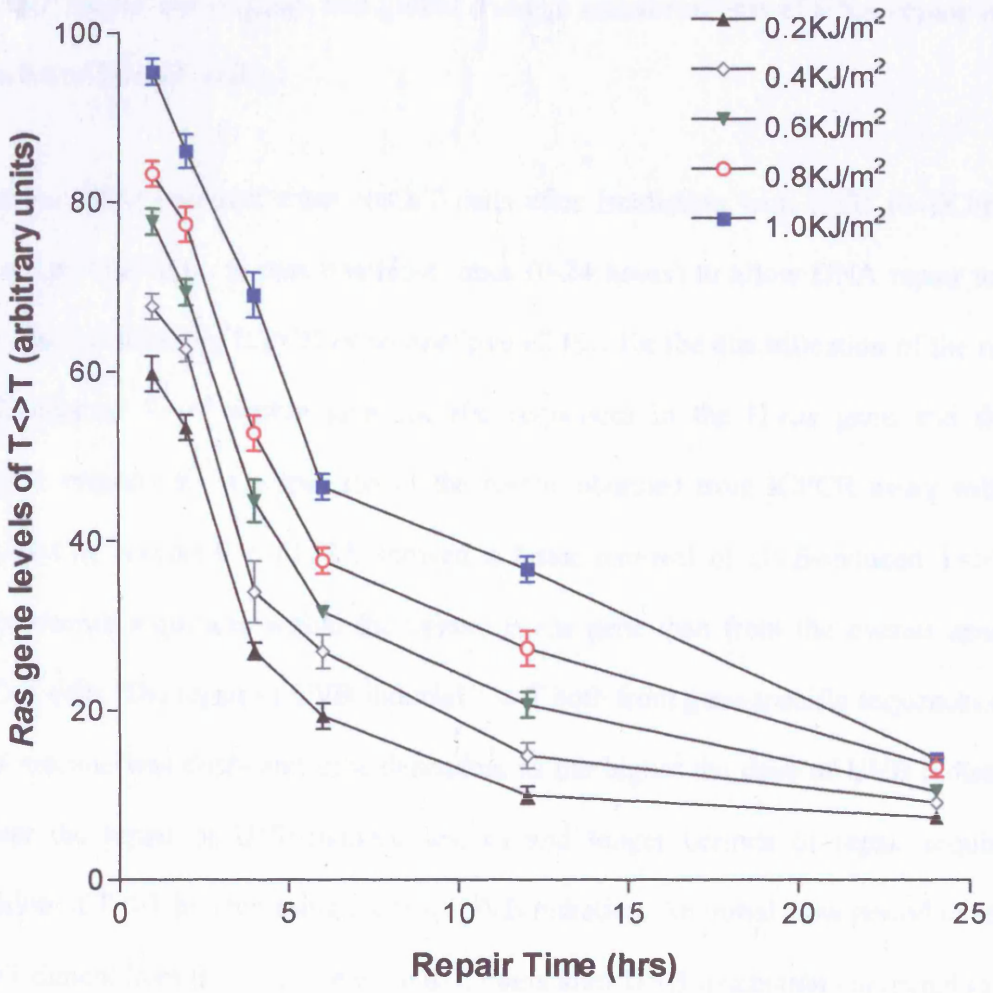


Figure 6.17: ICPCR quantification of UVB-induced T<>T repair in the H-*ras* gene. Genomic DNA samples, isolated from HaCaT cells after exposure to UVB radiation (0-1KJ/m^2) and for a period of repair (0-24 hours), were digested with *Bam*HI enzyme and 50ng digests were assayed by ICPCR. The relative peak intensities were determined for each band for all the UVB doses and plotted against corresponding repair times. Data represents the mean (±S.D.) of three experiments per UVB dose per time point.

6.4.10 Comparison of gene- and global damage measurements of DNA repair in UVB-irradiated HaCaT cells

Genomic DNA, isolated from HaCaT cells after irradiation with UVB (0-1KJ/m^2) and following incubation at post-treatment times (0-24 hours) to allow DNA repair to occur, was assayed either by ICPCR or competitive ELISA for the quantification of the repair of UVB-induced T<>T within gene-specific sequences in the H-*ras* gene and the total genome respectively. Comparison of the results obtained from ICPCR assay with those obtained by competitive ELISA showed a faster removal of UVB-induced T<>T from gene-specific sequences within the human H-*ras* gene than from the overall genome of HaCaT cells. The repair of UVB-induced T<>T both from gene-specific sequences and the total genome was dose- and time-dependent as the higher the dose of UVB radiation the slower the repair of UVB-induced lesions and longer periods of repair required for excision of T<>T by increasing doses of UVB radiation. An initial slow period of repair of T<>T dimers from the H-*ras* gene up to 2 hours after UVB-irradiation compared to that in the global genome was detected. Exposure of cells to 0.2 and 0.4KJ/m^2 of UVB radiation resulted in 47% and 37% of T<>T removal from the H-*ras* gene respectively compared to that in the global genome where for the same doses 57% and 47% of T<>T were removed after 2 hours of repair. However, a faster repair of T<>T was detected between 4 and 24 hours post-irradiation. After 6 hours of repair, 73% of T<>T were removed from the H-*ras* gene following exposure of cells to 0.4 KJ/m^2 of UVB radiation compared to that in the global genome where 66% of lesions were repaired at the same dose and within the same period of repair. By 24 hours, approximately 7% of T<>T were still present at the lowest UVB dose (0.2KJ/m^2) in gene-specific sequences within the human H-*ras* gene and about 21% in the global genome of the cells for the same UVB dose. A summary of the results obtained by ICPCR and competitive ELISA for the repair of UVB-induced T<>T in H-*ras*

gene and the total genome of HaCaT cells after 2, 6 and 24 hours of repair is shown in Table 6.2.

TABLE 6.2: Comparison of the repair of UVB-induced T<>T in gene-specific sequences within the H-*ras* gene and the total genome of HaCaT cells by ICPCR and competitive ELISA respectively. Data represents the mean (±S.D.) of three experiments per UVB dose per time point.

UVB-induced T<>repaired (%)

	2hrs		6hrs		24hrs	
UVB dose (KJ/m^2)	**Global genome**	**H-*ras* gene**	**Global genome**	**H-*ras* gene**	**Global genome**	**H-*ras* gene**
0.2	57 (± 0.807)	47 (± 0.882)	69 (±1.062)	80 (± 0.882)	79 (± 0.716)	93 (± 0.115)
0.4	47 (± 1.244)	37 (± 0.885)	66 (± 1.301)	73 (± 1.155)	77 (± 0.674)	91 (± 0.186)
0.6	43 (± 1.290)	31 (± 0.882)	58 (± 0.775)	68 (± 0.333)	75 (± 1.198)	89 (± 0.289)
0.8	37 (± 0.450)	23 (± 1.202)	56 (± 0.931)	62 (±0.884)	74 (± 0.481)	87 (± 0.667)
1.0	34 (± 1.124)	14 (± 1.155)	52 (± 0.755)	54 (± 0.88)	72 (± 0.790)	85 (± 0.333)

6.5 DISCUSSION

Ultraviolet radiation causes DNA damage, which results in the formation of potentially mutagenic lesions, such as UVR-induced cyclobutane pyrimidine dimers (CPDs), if left unrepaired. The heterogeneous distribution of DNA damage throughout the genome and preferential repair of actively transcribed genes *versus* inactive regions or the overall genome (Bohr *et al.*, 1985, 1987; Mellon and Hanawalt, 1989; Hanawalt, 1989), necessitates the need for quantification of DNA damage and repair in specific genomic sequences, as they are likely to have greater relevance to the disease end-point.

In this study, the induction of DNA damage and repair in gene-specific sequences within the human H-*ras* gene and the global genome of UVB-irradiated HaCaT cells was investigated using immuno-coupled PCR (ICPCR) assay (Denissenko *et al.*, 1996). Irradiation of HaCaT cells with UVB was of biological significance, given that the minimal erythema dose (MED) induced by UVB radiation in human skin is in the range of 100-1000J/m^2 (Harber and Bickers, 1989). A linear relationship between the amount of PCR product and the dose of UVB radiation was established using ICPCR assay. Cellular treatment with increasing doses of UVB radiation resulted in DNA samples with higher levels of UVB-induced T<>T to be preferentially captured by the antiserum IgG479, which was detected by an increase in the intensity of the PCR signal. This observation of a proportional relationship between the initial amount of damage and the resulting amount of PCR product agreed well with the results obtained from the quantification of UVC-induced DNA damage in human genomic DNA within 0 to 3.5KJ/m^2 of UVC radiation (previously described in Chapter 5). In addition, Denissenko *et al.* (1994) showed a linear induction of *anti*-BPDE adducts in human skin fibroblast cells after *in vivo* treatment with increasing doses (0-8μM) of *anti*-BPDE DNA genotoxin by polyclonal antibody BP-1. A dose-

dependent induction of CPDs in HaCaT cells after *in vitro* irradiation with UVB (0-1KJ/m^2) was also established by flow cytometry assay using a monoclonal antibody (Greinert *et al.*, 2000). Similarly, Van Houten *et al.* (2000) showed a linear increase of CPD induction in human fibroblast cells irradiated with UVC light by quantitative PCR.

Furthermore, amplification yields were limited only by template availability, as the amount of PCR product was shown to be proportional to the starting amount of DNA template. These results agreed with previous findings obtained from the quantification of UVC-induced DNA damage in human genomic DNA within 10 to 100ng of template DNA (previously described in Chapter 5). Also, work by Denissenko *et al.* (1994) demonstrated a linear relationship between the initial amount of template DNA and the resulting amount of PCR product within 1 to 25ng for genomic DNA isolated from human fibroblasts using quantitative immuno-coupled PCR.

Replicate experiments and analysis of PCR standards run in parallel in each PCR were performed in order to establish the reproducibility of ICPCR assay in a cellular system. Quantification of UVB-induced T<>T in discrete fragments of genomic DNA isolated from HaCaT cells was established using antiserum IgG479. The ability of this dimer specific antiserum to bind specifically T<>T contained within H-*ras* gene fragments derived from UVC-irradiated genomic DNA had been previously shown in Chapter 5. In addition, effective amplification of a 149bp target region within the human H-*ras* gene was achieved in each PCR expression allowing thus, the sensitive and accurate quantification of UVB-induced T<>T and their repair *in vivo*.

Induction of T<>T in UVB-irradiated HaCaT cells was established immunocytochemically by lesion-specific antiserum IgG479. A dose-dependent increase in the induction of T<>T within the nucleus of HaCaT cells was detected following exposure of cells to increasing doses of UVB radiation, suggesting the potential use of this antiserum as a DNA damage probe in the quantitative assessment of UVB-induced DNA damage *in vivo*. The induction of T<>T localised in the nucleus of human keratinocytes after exposure to 1.08KJ/m^2 of UVB radiation was previously shown by immunofluorescence microscopy using sequence-specific polyclonal antibody 529 (Cooke *et al.*, 2000b).

A highly significant, linear relationship was established from the comparison of UVB-induced T<>T dimers in gene-specific segments using ICPCR assay and in total genome of HaCaT cells by competitive ELISA. This proportional relationship between gene-specific and global measures of T<>T had been previously shown (Chapter 5) from the quantification of UVC-induced DNA damage with human genomic DNA. Similarly, Denissenko *et al.* (1994) noted good agreement between gene and global level repair rates of *anti*-BPDE adducts in XP group A fibroblasts.

Nucleotide excision repair (NER) prevents skin cancer by eliminating highly genotoxic cyclobutane pyrimidine dimers (CPDs) induced in DNA by the UVB component of sunlight (Sancar, 1994). Preferential repair of UVR-induced pyrimidine dimers from transcriptionally active genes than from non-transcribed regions of the genome has been shown by several studies (Bohr *et al.*, 1985; Mellon *et al.*, 1987; Mellon and Hanawalt, 1994). In this study, the repair of UVB-induced T<>T in gene-specific sequences within the human H-*ras* proto-oncogene and the total genome of HaCaT cells was established using ICPCR and competitive ELISA respectively. The removal of UVB-induced T<>T

both from specific gene fragments and the total genome occurred in a dose-dependent manner, with low doses of UVB radiation resulting in faster repair of UVB-induced lesions compare to high doses, where a slower repair rate of T<>T was detected. Exposure of cells to 0.2 and 0.4KJ/m^2 of UVB, for example, resulted in 80% and 73% of T<>T removal from the H-*ras* gene within 6 hours of repair respectively, compared to higher doses (0.8 and 1.0KJ.m^2), where for the same period of repair 62% and 54% of DNA lesions had been repaired respectively. This delay in the activity of NER pathway by increasing doses of UVB radiation agreed with the results obtained from exposure of normal human keratinocytes to similar doses of UVB, where an efficient removal of CPDs from the genome of keratinocytes after exposure to low doses of UVB was shown by a slot blot DNA repair activity assay (Maeda *et al.*, 2001). In addition, longer periods of repair were required for the excision of T<>T both from the H-*ras* gene and the total genome by increasing doses of UVB radiation. At 2 hours after irradiation, exposure of cells to 0.8KJ/m^2 showed 23% of T<>T repair compared to that of 6 hours where 62% of T<>T had been removed from the H-*ras* gene. These results agreed with those obtained from the measurement of DNA damage in HaCaT cells after *in vitro* irradiation with similar UVB doses by a flow cytometric assay, where the removal of CPDs from the genome of the cells was dose-dependent, with increasing values of time constants (τ = 11 hours for 100J/m^2 and τ = 35 hours for 800J/m^2) for increasing UVB doses (Greinert *et al.*, 2000). Furthermore, this observation of a dose-dependent reduction in the removal of T<>T from the genome of HaCaT cells was shown by the measurement of excised T<>T in the cell supernatants. Following exposure of cells to 0.8KJ/m^2 of UVB radiation, 64% of T<>T were excised in the cell culture medium while 83% of T<>T were removed from the cells at 0.2KJ/m^2 after 6 hours of irradiation. This delay in the repair of UVB-induced T<>T by increasing doses of UVB radiation could be due to an inhibition in the activity or

interruption in the synthesis of the enzymes involved in the NER pathway by UVB radiation (Grossweiner, 1976; Tallmadge *et al.*, 1990). Recent data by Greinert *et al.* (2000) showed that the dose-dependence decrease in the repair of UVB-induced CPDs in HaCaT cells could be due to the effects of UVB radiation on DNA repair enzymes, which are available in the G1 phase of the cell cycle, although more experiments need to be carried out to confirm this. ICPCR assay relies upon a proportional relationship between the initial dose of damage and the resulting amount of PCR product. Thus, the differences observed in the repair rate of UVB-induced T<>T in the H-*ras* gene were due to absolute amount of damage rather than differences in rate.

Viability of HaCaT cells following exposure to UVB radiation was performed using MTT assay (Mossman, 1983). A dose-dependent decrease in the survival rate of cells exposed to high doses of UVB radiation was detected compared to those treated with low doses. Exposure of cells to 0.2KJ/m^2 resulted in 60% cell survival at 24 hours post-irradiation compared to 44% after exposure to 0.8KJ/m^2 of UVB within the same period of time. A dose of 410J/m^2 of UVB was shown by flow cytometry assay to be sufficient to reduce viability of normal human keratinocytes to 37% (D_{37}) (Maeda *et al.*, 1994), suggesting apoptosis of cells following exposure to high doses of UVB radiation. Similarly, a D_{37} of 117J/m^2 of UVB radiation was shown in HaCaT cells by colony-forming assay (Greinert *et al.*, 2000). However, in this study, cellular treatment with 1000J/m^2 resulted in an approximately 40% of cell survival following 24 hours after UVB-irradiation. These differences in the numbers of viable cells after exposure to UVB radiation could be explained by the different UVB light sources and assays used to measure cell viability. Moreover, MTT assay was reported to measure cell proliferation (Mossman, 1983). Thus, it can be assumed that although, a reduction in MTT dye was detected by increasing UVB

doses, and due to the requirement of cell to cell contact for growth (Boukamp *et al.*, 1988), cells were still viable at high UVB doses but unable to proliferate. This hypothesis agreed with the results obtained from the flow cytometric measurements of the repair kinetics of HaCaT cells after exposure to similar UVB doses, where the cells were found to be viable and synchronized in the G1 phase of the cell cycle by contact inhibition without proliferating (Greinert *et al.*, 2000). However, because of the nature of the assay used in this study to measure cell viability, the location of the cells in the cell cycle was not possible. In addition, the low survival rates of HaCaT cells observed at high UVB doses caused no interference in the quantification of the repair of UVB-induced DNA damage by ICPCR and ELISA, as T<>T dimers were shown to be efficiently repaired in HaCaT cells by the NER pathway.

Comparison of the results obtained from ICPCR assay with those obtained by competitive ELISA showed a faster repair of UVB-induced T<>T from gene-specific sequences within the human H-*ras* gene compared to that from the overall genome of UVB-irradiated HaCaT cells. Following an initial slow period of T<>T repair up to 2 hours after UVB-irradiation, with 31% and 23% of T<>T removal from the H-*ras* gene after irradiation with 0.6 and 0.8KJ/m^2 of UVB respectively compared to that in the overall genome where for the same doses 43% and 37% of T<>T were removed within the same period of repair, a faster repair of T<>T was detected between 4 and 24 hours. After 6 hours post-irradiation, 80% and 73% of T<>T were removed from gene-specific sequences at 0.2 and 0.4 KJ/m^2 of UVB radiation respectively compared with those in the overall genome where 69% and 66% of lesions were repaired at the same doses and within the same period of repair respectively. This observation of an initial slow repair of DNA damage agreed with the results obtained from the measurement of the repair of UVC-induced DNA damage in the

dihydrofolate reductase (DHFR) gene segment in mouse leukaemia cells by quantitative PCR (Kalinowski *et al.*, 1992), where less than 10% of UVC-induced DNA lesions were removed after 4 hours of UVC-irradiation and over 70% of the lesions were removed by 8 hours post-irradiation. However, these results were in contrast to those obtained from the measurement of the repair of *anti*-BPDE adducts in human fibroblast cells, where about 60% of adducts were removed from the H-*ras* gene within the first hour followed by a slower repair of lesions of about 80% in the next 17 hours of repair (Denissenko *et al.*, 1994). The initial slow period of repair of UVB-induced T<>T from the H-*ras* gene could be due to a slower repair rate of enzymatically induced breaks occurred during DNA repair (Kalinowski *et al.*, 1992). Faster repair of these breaks by time resulted thus, in faster removal of UVB-induced DNA damage from the H-*ras* gene by the NER pathway within longer periods of repair.

Following 24 hours post-irradiation, 93% of UVB-induced T<>T were removed from the H-*ras* gene after exposure to 0.2KJ/m^2 of UVB radiation compared to 79% of lesion removal from the overall genome of HaCaT cells after exposure to the same UVB dose and within the repair time. The faster repair of UVB-induced T<>T from the H-*ras* gene than from the overall genome of HaCaT cells agreed with the results obtained from the measurement of the repair of UVC-induced DNA damage in Chinese hamster ovary cells (CHO) by Southern hybridisation based assay using T4 endonuclease V, where 70% of UVC-induced pyrimidine dimers were preferentially removed from the active DHFR gene compared to 15% from the overall genome of the cells (Bohr *et al.*, 1985). In addition, Denissenko *et al.* (1994) showed preferential repair of *anti*-BPDE induced adducts from the transcriptionally active H-*ras* proto-oncogene with about 80% of adduct removal within 18 hours of repair compared to 58% from the overall genome of human fibroblasts

within the same time of repair. The presence of UVB-induced T<>T dimers in HaCaT cells after 24 hours of repair agreed with the results obtained from the measurements of the repair of UVB-induced CPDs in the genome of HaCaT cells by flow cytometry, where about 9% of CPD lesions were still present in the genome of HaCaT cells following 168 hours of repair (Greinert *et al.*, 2000). Also, Young *et al.* (1996), showed unrepaired T<>T dimers in human skin types I and II following *in situ* irradiation with solar-simulating light after 7 days of repair. Furthermore, Denissenko *et al.* (1994) showed that about 42% and 20% of *anti*-BPDE adducts were not repaired in the overall genome of human fibroblast cells and in the H-*ras* gene within 18 hours of repair respectively.

In summary, ICPCR was found to be a suitable approach for the sensitive and quantitative assessment of UVB-induced DNA damage and repair in gene-specific sequences within the human H-*ras* gene. A dose-dependent increase in the induction and repair of UVB-induced T<>T both for the human H-*ras* gene and the overall genome of the HaCaT cells was detected by polyclonal antiserum IgG479. Using ICPCR assay, measures of UVB-induced T<>T in the H-*ras* gene were found to be proportional to measures of lesions in the overall genome. However, the results obtained from the quantification of the repair of DNA damage by ICPCR showed preferential repair of UVB-induced T<>T for the H-*ras* gene than from the overall genome of HaCaT cells, suggesting the potential for this assay in the quantitative measurement of gene-specific repair of various genotoxic lesions.

CHAPTER 7: GENERAL DISCUSSION

CHAPTER 7

GENERAL DISCUSSION

Clinical, experimental and epidemiological studies have implicated the ultraviolet component of solar radiation as the major determinant in the development of skin cancer due to its potential to damage DNA leading thus, to the generation of potentially mutagenic lesions in the DNA sequence and eventually the development of cancer (Peak and Peak, 1989; Ananthaswamy and Pierceall, 1990). DNA may be damaged either directly by absorption of UVR photons, leading to the formation of lesions, such as CPDs and (6-4)PPs (Cadet and Vigny, 1990), or indirectly *via* reactive oxygen species generating oxidative DNA lesions, such as 8-oxodG (Tyrrell and Kvam, 1997). Potentially important genes for the induction of DNA damage by UV radiation are the *ras* proto-oncogenes and the *p*53 tumour suppressor gene (Tornaletti *et al.*, 1993). Mutations in the *p*53 gene have been shown to occur in high frequency in UVR-induced skin malignancies, such as BCCs and SCCs, in the form of C→T transitions and CC→TT double transitions at dipyrimidine sequences, which are specifically induced by UV light and known as UV 'signature' mutations (Brash *et al.*, 1991; Ziegler *et al.*, 1993). Faithful maintenance of the integrity of the genome and accurate transmission of genetic information by DNA is crucial to all living organisms. Cells have, therefore, developed efficient DNA repair mechanisms, such as excision repair pathways (BER, NER and TCR), to counteract the mutagenic effects of UVR-induced and oxidative DNA damage (Sancar, 1995; Wood, 1996).

Several methods have been developed for the assessment of UVR-induced DNA damage, including ^{32}P-postlabelling (Bycov *et al.*, 1995), HPLC (Cadet *et al.*, 1983), GC-MS

(Podmore *et al.*, 1996), HPLC with tandem mass spectrometry (Douki *et al.*, 2000), HPLC with electrochemical detection (Floyd *et al.*, 1986). Though important, all these techniques have limitations, perhaps most significantly the inability to localise DNA damage in specific genes. This is very important, since studies on carcinogens, such as UV radiation, have demonstrated the induction of DNA damage and repair to occur in a non-random manner throughout the mammalian genome (Bohr *et al.*, 1987). UVR-induced CPDs, for example, have been shown to be preferentially removed from active genes than in inactive regions or the overall genome (Bohr *et al.*, 1985; Mellon and Hanawalt, 1989). This rapid repair was shown to be due to a faster repair of damage in the transcribed strand than in the non-transcribed strand (Mellon *et al.*, 1987; Leadon and Lawrence 1991). Faster repair of DNA lesions located on the transcribed strand may also be due to their accessibility to repair proteins and the coupling of DNA repair with other cellular processes, such as transcription (Bohr *et al.*, 1987; Mellon and Hanawalt, 1989). This heterogeneity of DNA damage and repair necessitates the need for the employment of methods suitable for the detection and quantification of DNA damage and repair in individual genes, as further information on DNA damage and repair induced by various carcinogens might be obtained.

Detection and quantification of DNA damage and repair in specific gene sequences has been established using PCR-based assays (Govan *et al.*, 1990; Jennerwein and Eastman, 1991; Kalinowski *et al.*, 1992). However, though very sensitive, large numbers of lesions in the target DNA region are required in order to obtain a quantifiable decrease in amplification. Conversely, antibodies raised against DNA lesions have been successfully applied to the detection and quantification of DNA damage and repair within discrete target sequences using sequence-specific ALISS (adduct levels in specific sequences)

assay (Hochleitner *et al.*, 1991; Thomale *et al.*, 1994a) and immuno-coupled PCR (ICPCR) (Denissenko *et al.*, 1994; 1996b).

In this study, the ability of polyclonal antiserum IgG479 to detect sequence-specific DNA modifications induced by UVC radiation of DNA was investigated, thereby, extending the findings of Ahmad *et al.* (1999). Competition experiments showed adjacent pyrimidines, particularly thymines, were essential for effective inhibition, as was originally observed by Levine *et al.* (1966). Data obtained from the use of short sequences of pyrimidines flanked by adenine residues suggested a minimum of three adjacent thymines to be the important epitope recognised by this antiserum. This observation is in agreement with previous studies on antisera raised against UVC-DNA using oligonucleotides with thymine residues (Herbert *et al.*, 1994; Ahmad *et al.*, 1999). In addition, results obtained from competitive ELISA using UVC-irradiated oligonucleotides with a 5' and a 3' cytosine demonstrated that cytosines could partially replace thymines within the smallest effective epitope 5' or 3' to T<>T, but not as a C<>T dimer. Thus, inhibition was increased according to the location of the third pyrimidine relative to the dimer (TTT>TTC>CTT), which is in agreement with previous work on the characterisation of sequence specificity of antisera raised against UVC- and UVA-irradiated DNA (Herbert *et al.*, 1994).

The ability of the antiserum to bind T<>T within both UVC- and UVB-irradiated DNA was further investigated using competitive ELISA. The results showed a dose-responsive binding of antiserum both to UVC- and UVB-irradiated DNA over the lowest doses (0-3.48KJ/m^2) followed by saturation in antiserum's binding to both types of UV radiation. These results agreed with those obtained from the assessment of DNA damage after exposure to UVC and UVB radiation by a polyclonal antiserum, where the induction of

T<>T levels reached a plateau level at the highest dose (0.875mJ/m^2) for both types of UV radiation (Cooke *et al.*, 2003b).

The applicability of polyclonal antiserum IgG479 to recognise the induction and repair of two important DNA lesions, such as T<>T and 8-xodG, in the urine of patients with guttate or plaque psoriasis following treatment with UVB phototherapy was investigated using a non-invasive immunoassay (ELISA). When compared to controls and corrected for creatinine and thus, urine concentration, results obtained from competition ELISA showed increased levels of T<>T in the urine samples of psoriasis patients recognized as repaired products of the NER pathway by the polyclonal antiserum. Increases in urinary levels of T<>T were noted for a number of days post-irradiation and is consistent with other studies, postulated to be due to a delay between lesion induction, repair, processing and appearance in the urine (Cooke *et al.*, 2001b). On the contrary, no increase in the levels of urinary 8-oxodG was observed at any time post-irradiation, in contrast to previous findings with a UVA source as part of psoralen plus UVA treatment (PUVA) (Cooke *et al.*, 2001b). From this it may be concluded that the therapeutic emission peak at 311nm of the TL-01 sources used in NB-UVB phototherapy, whilst effective at inducing T<>T, precludes the formation of oxidative DNA lesions, such as 8-oxodG, minimising thus, the cancer risk often associates with the use of the UVA component of PUVA treatment. Biomonitoring, therefore, of phototherapies used in treatment of psoriasis with antibodies may result in an improved evaluation of the risks involved and assist in a better understanding of the potential mechanism through which phototherapies exert their beneficial and side-effects.

Antibody-based assays have been successfully employed to quantify DNA base modifications induced by various genotoxic agents (Wani and D'Ambrosio, 1987; Wani

and Arezina, 1991; Denissenko *et al.*, 1994, 1996b). In this study, a sensitive and semi-quantitative approach was developed to measure DNA damage and repair within specific human genes. The ICPCR assay relies upon proportionality between the amount of PCR product and the initial amount of damage, previously demonstrated by Denissenko *et al.* (1994). The linear relationship between the dose of UVC-and UVB-radiation and resulting amount of PCR product, demonstrated that the polyclonal antiserum IgG479 was able to specifically bind to T<>T contained within H-*ras* gene fragments derived from UVC-irradiated genomic DNA and DNA isolated from UVB-irradiated human keratinocyte cells (HaCaT). This observation of a linear relationship between the initial amount of damage and the resulting amount of PCR product agreed with those obtained from the immunocapture of genomic DNA from human fibroblast cells, after *in vivo* treatment with increasing doses of *anti*-BPDE DNA genotoxin by polyclonal antibody BP-1 (Denissenko *et al.*, 1994). A dose-dependent induction of CPDs in HaCaT cells after *in vitro* irradiation with UVB (0-1KJ/m^2) was also established by flow cytometry assay using a monoclonal antibody (Greinert *et al.*, 2000), and by quantitative PCR after irradiation of human fibroblast cells with UVC light (Van Houten *et al.*, 2000). This is essential to the quantitative measurement of gene-specific DNA damage and repair (Bohr, 1991). Furthermore, a dose-dependent increase in the induction of T<>T within the nucleus of UVB-irradiated HaCaT cells was detected immunocytochemically by antiserum IgG479, suggesting its potential use as a DNA damage probe for the quantitative assessment of UVB-induced DNA damage *in vivo*. Induction of T<>T localised in the nucleus of human keratinocytes after exposure to UVB radiation was previously shown by immunofluorescence microscopy using sequence-specific polyclonal antibody 529 (Cooke *et al.*, 2000b), but without a clear dose-effect.

ICPCR assay was demonstrated to be able to detect UVC-induced damage in gene-specific sequences at a UVC dose of as low as $3J/m^2$. Although, such a dose might still be lethal for cells, as a lethal dose of UVC radiation is about $2.5J/m^2$ (Van Houten *et al.* 2000), it does approximate to doses used by other, similar, techniques such as LMPCR ($10J/m^2$; Gao *et al.*, 1994) and only a 0.5-fold higher than the QPCR assay where Van Houten *et al.* (2000) described the use of a long PCR to measure doses of UVC radiation down to $2.5J/m^2$. A relatively high dose ($24KJ/m^2$) of UVC radiation has been used in the measurement of DNA repair in 147-440bp fragments *in vivo* (Govan *et al.*, 1990). On the contrary, a low dose of UVC radiation ($2.5KJ/m^2$) was used in the measurement of gene-specific repair in human cells (Van Houten *et al.*, 2000) but in PCR fragments as long as 24 kb. Similarly, Wani and Arezina (1991) demonstrated the measurement of DNA damage and repair within larger fragments (>5kb length) of plasmid DNA by UVC doses as low as 1 to 2 J/m^2.

PCR primers used in this study were reported to amplify of the 149bp target region in a 6.4kbp H-*ras* gene fragment (Denissenko *et al.*, 1994). Only nanogram amounts of DNA were required to achieve specific immunocapture and quantification. In addition, amplification was limited only by template availability, as the amount of the PCR product was shown to be proportional to the initial amount of template DNA. The results obtained by ICPCR assay agreed with those obtained from the immunocapture of genomic DNA from human fibroblasts, treated *in vivo* with *anti* BPDE genotoxin, by BP1 antibody (Denissenko *et al.*, 1994), where a linear relationship was shown for the initial amount of template DNA and the resulting amount of PCR product within 1-25ng for genomic DNA.

Replicate experiments and analysis of PCR standards run in parallel in each PCR were performed in order to establish the reproducibility of the assay. The effective amplification of 149bp fragment in each PCR and the non-existence of primer dimer formation made the use of the PCR feasible without the requirement of an internal standard, essential in relative analysis. The thermal cycling profile (30 cycles) used in ICPCR for amplification of specific-gene sequences within the human H-*ras* proto-oncogene was found to be sufficient for reproducible amplification of small amounts (nanograms) of template DNA. The conditions used in the amplification of the target DNA template where within the exponential phase of the reaction as determined by real-time QPCR.

A highly significant, linear relationship was established from the correlation of measures of UVC-induced T<>T in gene-specific segments using ICPCR assay and in total genome using both GC-MS and competitive ELISA, indicating that gene-specific measures of lesions are proportional to global measures of T<>T. Similarly, a highly significant linear relationship between measures of T<>T in the H-*ras* gene and the total genome of UVB-irradiated HaCaT cells was demonstrated by ICPCR and competitive ELISA, respectively. This result was unexpected, given that induction of DNA damage occurs heterogeneously throughout the genome. Similarly, Denissenko *et al.* (1994) noted good agreement between gene and global level repair rates within their cellular system. However, as the comparisons were performed using extracted DNA, in the absence of repair enzymes, or nucleosomal proteins, the distribution of damage is likely to be much more homogeneous, which is supported by the similar responses noted with both GC-MS and competitive ELISA.

Preferential repair of UVR-induced pyrimidine dimers from transcriptionally active genes than from non-transcribed regions of the genome has been previously shown by several studies (Bohr *et al.*, 1985; Mellon *et al.*, 1987; Mellon and Hanawalt, 1994). The repair of T<>T in gene-specific sequences within the human H-*ras* proto-oncogene and the total genome of UVB-irradiated HaCaT cells was established using ICPCR and competitive ELISA, respectively. Repair of UVB-induced T<>T by the NER pathway both from the H-*ras* gene and the total genome occurred in a dose-dependent manner, with lower doses of UVB radiation resulting in faster removal of DNA lesions compared to higher ones. This delay in the activity of NER pathway by increasing doses of UVB radiation agreed with the results obtained from exposure of normal human keratinocytes to similar doses of UVB, where an efficient removal of CPDs from the genome of keratinocytes after exposure to low doses of UVB was shown by a slot blot DNA repair activity assay (Maeda *et al.*, 2001). In addition, longer periods of repair were required for the excision of T<>T both from the H-*ras* gene and the total genome by increasing doses of UVB radiation. These results are in agreement with those obtained from the measurement of DNA damage in HaCaT cells after *in vivo* irradiation with similar UVB doses by a flow cytometric assay, where the removal of CPDs from the genome of the cells was dose-dependent, with increasing values of time constants for increasing UVB doses (Greinert *et al.*, 2000). This finding of a dose-dependent reduction in the removal of T<>T from the genome of HaCaT cells was further supported by the measurement of UVB-induced T<>T in the supernatants of the cells. This delay in the repair of UVB-induced T<>T by increasing doses of UVB radiation could be due to an inhibition in the activity or interruption in the synthesis of the enzymes involved in the NER pathway by UVB radiation (Grossweiner, 1976; Tallmadge *et al.*, 1990). In addition, Greinert *et al.* (2000) showed that the dose-dependence decrease in the repair of UVB-induced CPDs in HaCaT cells could be due to the effects of UVB

radiation on DNA repair enzymes, which are available in the G1 phase of the cell cycle, although more experiments are required to refine this observation.

Following an initial slow period of repair of UVB-induced T<>T up to 2 hours, a faster repair of T<>T from the H-*ras* gene compared to the global genome was observed between 4 and 24 hours post-irradiation by ICPCR and competitive ELISA, respectively. This observation of an initial slow repair of DNA damage agreed with the results obtained from the measurement of the repair of DNA damage in mouse leukaemia cells after exposure to UVC-irradiation by quantitative PCR (Kalinowski *et al.*, 1992), where less than 10% of UVC-induced DNA lesions were removed after 4 hours of UVC-irradiation and over 70% of the lesions were removed by 8 hours post-irradiation. On the contrary, Denissenko *et al.* (1994) observed a faster repair of *anti*-BPDE adducts in human fibroblast cells within the first hour followed by a slower repair of lesions in the next 17 hours of repair (Denissenko *et al.*, 1994). The initial slow period of repair of UVB-induced T<>T from the H-*ras* gene could be due to a slower repair rate of enzymatically induced breaks occurred during DNA repair (Kalinowski *et al.*, 1992), whereas a faster repair of these breaks by time resulted in faster removal of UVB-induced DNA damage from the H-*ras* gene by the NER pathway.

Preferential repair of UVB-induced T<>T from the transcriptionally active H-*ras* gene compared to the overall genome of human HaCaT cells was observed and is consistent with the data of Denissenko *et al.* (1994). However, residual UVB-induced DNA damage was detected in the HaCaT cell after 24 hours of repair and is in agreement with the results obtained from the measurements of the repair of UVB-induced CPDs in the genome of HaCaT cells by flow cytometry, where UVB-induced lesions were still present 168 hours after irradiation of human HaCaT cells (Greinert *et al.*, 2000). Also, Young *et al.* (1996),

showed unrepaired T<>T dimers in human skin types I and II following *in situ* irradiation with solar-simulating light after 7 days of repair. Furthermore, Denissenko *et al.* (1994) showed that the presence of *anti*-BPDE adducts in the overall genome of human fibroblast cells and in the H-*ras* gene within 18 hours of repair.

To sum up, ICPCR assay was found to be a suitable approach for the sensitive and quantitative assessment of UVC- and UVB-induced DNA damage and repair in gene-specific sequences within the human H-*ras* gene, suggesting the importance of this assay in the quantitative measurement of gene-specific DNA damage and repair of various genotoxic lesions. The specificity of polyclonal antiserum IgG479 to bind to T<>T induced by both UVC- and UVB-irradiation within discrete target sequences, makes it an attractive tool to the assessment of gene-specific DNA damage and repair of a potentially mutagenic DNA lesion, such as cyclobutane thymine dimer.

CONCLUSIONS

Over the last two decades studies on the induction of DNA damage and repair by various DNA-damaging agents, including UV radiation, have shown heterogeneity of the DNA repair processes in the mammalian genome. The discovery of preferential repair of active genes following exposure to UV radiation has provided the basis of understanding the fine structure of DNA repair, which often does not appear when DNA repair is studied with more traditional approaches where events are averaged over the entire genome.

In this study:

- A polyclonal antiserum, which has the specificity to recognise UV damaged DNA containing T<>T, was successfully used to detect sequence-specific DNA modifications after exposure to UV radiation.

- Application of this polyclonal antiserum in the detection of T<>T and 8-oxodG, recognised as repaired products of the NER pathway, in the urine of psoriatic patients following treatment with NB-UVB phototherapy, showed increased levels of T<>T whereas no increase in the of 8-oxodG levels was observed at any time post-irradiation.

- Quantification of T<>T in naked DNA following UVC irradiation was established using a sensitive approach, immuno-coupled PCR (ICPCR), employing the polyclonal antiserum specific for T<>T. The induction of UVC-induced T<>T within gene-specific sequences in the human H-*ras* proto-oncogene was shown to occur in a linear dose-responsive manner. Using, this assay, a highly significant

linear relationship was established from the correlation of T<>T measures in gene-specific segments and in the total genome using both GC-MS and ELISA assay.

- Application of ICPCR in the assessment of DNA damage and repair in human keratinocytes following exposure to UVB radiation demonstrated a dose-dependent increase in the induction of and repair of T<>T both for the human H-*ras* gene and the global genome of keratinocytes by polyclonal antibody. Comparison of measures of UVB-induced T<>T in the H-*ras* gene were found to be proportional to measures of lesions in the overall genome. However, UVB-induced T<>T were preferentially repaired from the transcriptionally active H-*ras* proto-oncogene than from the overall genome of the human keratinocytes as it was determined by ICPCR.

In summary, these data demonstrated that ICPCR assay is a suitable approach for the quantitative measurement of DNA damage and repair at the level of a gene, allowing correlations between specific genes and total genome measures of DNA damage and repair to be performed. The ultimate goal of future studies in the area of damage and repair to DNA should therefore, be the development of methods, which could be able to detect and measure the damage formation and repair for a number of carcinogens and chemotherapeutics in individuals genes. Such methods would hopefully provide insight into the aetiology and treatment of malignancies.

FUTURE WORK

It is now clear that quantification of DNA damage and repair in defined gene sequences is of particular importance, as it might provide a better insight into the molecular mechanisms of UVR-induced skin carcinogenesis in humans.

The present study has demonstrated the application of a sensitive immunoassay, immuno-coupled polymerase chain reaction (ICPCR), to the quantification of DNA damage and repair in specific genomic sequences within the human H-*ras* proto-oncogene following exposure to UVC- and UVB-radiation. A comparison analysis between measures of T<>T induced in the human H-*ras* gene and the global genome and the rates of the repair of T<>T was established by ICPCR and ELISA assays using a polyclonal antiserum specific for T<>T. The simplicity of ICPCR as not expensive equipment or reagents are required minimising thus, the cost and laborious work and the fact that quantification of UVR-induced DNA damage and repair was performed using small amounts (nanograms) of template DNA, makes ICPCR assay an attractive tool to the study of DNA damage and repair induced by various DNA adducts in individual genes recognised by specific antibodies for these adducts.

Future work will include application of ICPCR assay in the assessment of DNA damage and repair in another important gene in UVR-induced skin cancer, the *p*53 tumour suppressor gene. The *p*53 tumour suppressor gene serves an excellent model to study the development of sunlight-induced skin carcinogenesis in humans due to the high frequency of C→T and CC→TT mutations in patients with skin malignancies, such as SCC and BCC (Brash *et al.*, 1991; Ziegler *et al.*, 1993). A series of experiments similar to those for the

human H-*ras* gene would be performed in order to study the induction of DNA damage and repair in the *p*53 gene following irradiation of human keratinocytes with UVB. Levels of T<>T formed in gene-specific sequences and the total genome would be assayed by ICPCR and ELISA assay, respectively, allowing thus, a comparison between the two genes regarding the formation of T<>T and their repair rates both in target genes and the total genome.

The development of a polyclonal antiserum able to specifically bind to cytosine-cytosine dimers induced within discrete target sequences after exposure to UV radiation would be a second future step to the study of DNA damage and repair in specific genes. Production of such an antibody against UVC-irradiated poly(dC) would be performed according to the method described by Plescia *et al.* (1964) for the preparation of the immunogen [UVC-poly(dC) complexed with methylated BSA]. Immunization of the animal would be achieved according to the method described by Cooke *et al.* (1997) and would continue until sufficient discrimination between unirradiated and UVC-irradiated antigen has been established. In order to ensure effective production of C<>C within UVC-irradiated DNA a ketone triplet photosensitiser, such as acetone, would be used as it was previously described in the literature for the preparation of T<>T and C<>C dimers, respectively (Varghese, 1970; 1971). Data from various experiments showed formation of both thymine and cytosine derivatives and related nucleosides by acetone sensitisation through energy transfer in oxygen-free solutions (Vargese, 1970; Liu and Yang, 1978). The antigenic specificity of this antibody would be tested using various oligonucleotides as it was previously described for the characterisation of the polyclonal antiserum IgG479 by ELISA assays, both direct and competitive. Application of this antibody in the quantification of DNA damage and repair in gene-specific sequences within the human H-

ras proto-oncogene would be performed as previously described for the UVB-irradiated human cultured keratinocyte cells using ICPCR assay.

Development of an antibody to C<>C would be of particular importance as it would allow detection of DNA damage formed at CC sequences within DNA, and could be possibly used as a useful predictor of risk for susceptibility to mutation and overall risk for the development of skin cancer. This is true, particularly since the formation of T<>T, through its high induction in DNA following exposure to UV radiation, appears to be less mutagenic compared to that of C<>C, probably due to their correct replication bypass by DNA polymerase η (Yu *et al.*, 2001). The major mutagenic step in UV mutagenesis has been shown to occur by deamination of cytosine in CPDs (Tessman *et al.*, 1992) rather by a polymerase error. Deamination of cytosine results in the formation of C → T transition mutations at dipyrimidine sequences (Pfeifer, 2000). Indeed, these UV-induced transition mutations have been shown to result from correct DNA polymerase bypass of CPDs containing deaminated cytosine or 5-methylcytosine (Tu *et al.*, 1998; You and Pfeifer, 2001). Deamination of cytosine and 5-methylcytosine has been shown to occur at significant rates *in vivo* (Tu *et al.*, 1998), suggesting a possible role in the development of UVB-mutagenesis in humans. In addition, Tommasi *et al.* (1997) reported that dipyrimidines containing 5-methylcytosine to be preferential targets for sunlight action.

An interesting future step in the study of DNA damage and repair could be the application of DNA microarray technology to the study of the expression of genes in response to a carcinogen, such as UV radiation. This could be achieved by obtaining quantitative and qualitative information regarding DNA damage and repair in specific genes at a global genomic level. DNA microarray technology represents a breakthrough in molecular

biology in providing an unprecedented opportunity to explore the biological processes in a variety of organisms, including humans. The technology of DNA microarrays is powered by the development and integration of two key technologies, including the fabrication of numerous polynucleotides at high spatial resolution in precise locations on a surface and the laser confocal fluorescence scanning to measure molecular binding events on the array (Lipshutz *et al.*, 1999).

The developing technology of DNA microarrays provided scientists with a promising tool to examine a large portion of the transcriptome in a single experiment, allowing them to establish associations between characteristic gene expression patterns and molecular responses to drug therapy (Zhou *et al.*, 2002; Patel *et al.*, 2002; Donald *et al.*, 2002; de Longueville *et al.*, 2002) and characterise changes in pathological states within a tissue (Anzick and Trent, 2002; Kipps, 2002) as well as the development of expanded diagnostic protocols (Dobrowolski *et al.*, 1999; Guzey and Spigset, 2002; Ernst *et al.*, 2002).

A possible proposed method for the analysis of gene expression in the whole genome would involve exposure of human cultured keratinocytes to a dose range of UVB radiation and incubation of cells at different periods of time to allow repair to occur. This would then follow by extraction of DNA, fragmentation and immunocapture of damaged DNA fragments by a lesion specific antibody. Following amplification of DNA fragments containing-lesion and labelling of the DNA amplicon, hybridisation on a DNA Genechip array would then be performed. Using specific antibodies against DNA lesions of interest it might be possible to identify genes that have been selectively damaged. Validation of the results would then be achieved using Northern or dot blots or real-time quantitative PCR.

Analysis of global changes of gene expression in human epidermal keratinocytes following exposure to UV radiation might provide an explanation of the possible role of UV radiation in skin carcinogenesis, metabolic dysfunction and failure to arrest cell growth. The results of such an experiment will then be correlated with those from gene expression studies in order to obtain a better understanding of the pathological pathways involved in the response of cells to UVR-induced DNA damage. The analysis of human skin keratinocyte cells, for example, after irradiation with UVB might explain the development of skin carcinoma. It might also allow systematic study of mutagens, aging related DNA damage and cancer.

Previous gene expression studies in human keratinocytes have shown a generalised-down regulation of genes implicated in cell-adhesion and metabolism while a global induction of genes involved in pathological conditions, including stress-response, inflammation, apoptosis, mRNA splicing, replication and translation was observed (Sesto *et al.*, 2002). In addition, Dazard *et al.* (2003) provide information about the repair mechanisms and genes that have been damaged or modified their normal functions in response to UVB radiation in his comparison studies between normal human epidermal keratinocytes and squamous cell carcinoma cells. Furthermore, due to the heterogeneity of DNA damage induction and repair with preferential removal of lesions from actively expressed genes compared to inactive genes or the overall genome (Bohr *et al.*, 1985), the susceptibility of inactive genes in response to UVR-induced DNA damage could be investigated by using gene expression studies, which would allow a possible comparison with the actively expressed genes, which might have more efficient repair mechanisms. To sum up, DNA microarray technology might provide information about the complexity of the transcriptional profile of genes in response to UVB radiation, several cellular processes previously not known to be

affected by UV irradiation, and serve as a basis for the global characterization of UV-regulated genes and pathways.

Ultraviolet radiation experiments have made significant contributions to biology. They were amongst the first to reveal the importance of nucleic acids in cell function and reproduction, and they are presently contributing to knowledge of the nature of mutation. The measurement of UVR-induced DNA damage and repair in specific genomic sequences will hence add immeasurably to the understanding of the vital role of ultraviolet radiation in the induction of skin cancer in humans.

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Appendix I Publications arising from this thesis

Karakoula, A., Cooke, M. S., Evans, M. D., Hutchinson, P. E. and Lunec, J. (2002) Gene-specific measurements of DNA damage-a better biomarker? *Free Radical Biology and Medicine* **33:** S56.

Karakoula, A., Evans, M. D., Podmore, I. D., Hutchinson, P. E., Lunec, J. and Cooke, M. S. (2003) Quantification of UVR-induced DNA damage: global- versus gene-specific levels of thymine dimers. *Journal of Immunological Methods* **277:** 27-37.

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Karakoula, A., Cooke, M. S., Evans, M. D., Hutchinson, P. E. and Lunec, J. (2002) Gene-specific measurements of DNA damage-a better biomarker? *Free Radical Biology and Medicine* **33:** S56.