

## Wavelength dependence of oxidative DNA damage induced by UV and visible light

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**DNA damage induced by UV radiation and visible light (290–500 nm) in AS52 Chinese hamster cells was analysed by an alkaline elution assay with specific repair endonucleases. Cells were exposed to extensively filtered monochrome or broad-band radiation. Between 290 and 315 nm, the ratio of base modifications sensitive to Fpg protein (i.e. 8-hydroxyguanine and formamidopyrimidines) and T4 endonuclease V (i.e. cyclobutane pyrimidine dimers) was constant (~1:200), indicating that the direct excitation of DNA is responsible for both types of damage in this range of the spectrum. While the yield of pyrimidine dimers per unit dose continued to decrease exponentially beyond 315 nm, the yield of Fpg-sensitive modifications increased to a second maximum between 400 and 450 nm. The damage spectrum in this wavelength range consisted of only a few other modifications (strand breaks, abasic sites and pyrimidine modifications sensitive to endonuclease III) and is attributed to endogenous photosensitizers that give rise to oxidative DNA damage via singlet oxygen and/or type I reactions. The generation of Fpg-sensitive modifications by visible light was not linear with dose but followed a saturation curve. It is calculated that the exposure of the cells to low doses of solar radiation results in the formation of cyclobutane pyrimidine dimers and Fpg-sensitive modifications in a ratio of 10:1.**

### Introduction

Solar radiation can give rise to cellular DNA damage by (i) direct excitation of DNA and (ii) indirect mechanism(s) that involve the excitation of other cellular chromophores (endogenous photosensitizers) (1,2). The direct excitation of DNA generates predominantly cyclobutane pyrimidine dimers and (6–4) photoproducts, which are without doubt of principal importance for the cytotoxic, mutagenic and carcinogenic effects of short-wave UV radiation (UVC, UVB) (3–7). On the other hand, indirect mechanisms must be responsible for DNA damage and genotoxic effects observed at longer wavelengths (UVA, visible light), at which DNA absorbs only weakly or not at all (8,9). In this range of the spectrum, various oxidative DNA modifications such as 8-hydroxyguanine (8-oxoG\*), strand breaks, sites of base loss and DNA-protein crosslinks are generated (10–12). The mutagenic potential of some of these lesions, e.g. 8-oxoG, has been well

\*Abbreviations: 8-oxoG, 8-hydroxyguanine; Fpg protein, formamidopyrimidine-DNA glycosylase; MMS, methyl methanesulfonate; Fapy-A, 4,6-diamino-5-formamidopyrimidine.

established (13,14). Therefore, it is not surprising that the wavelength dependence of mutation induction by UV does not correlate with that of pyrimidine dimer formation (15) and that the action spectra obtained for photocarcinogenesis (16,17) indicate (or are at least compatible with) a role of non-dimer modifications in skin cancer induction.

The mechanisms involved in the generation of oxidative DNA modifications, in particular the specific absorbing chromophore(s) and the reactive species involved, remain to be established. In principle, an excited endogenous photosensitizer molecule could react with DNA without further intermediates (type I reaction) or via singlet oxygen (<sup>1</sup>O<sub>2</sub>) (type II reaction) (Figure 1). Alternatively, hydroxyl radicals (<sup>•</sup>OH) — generated via superoxide (O<sub>2</sub><sup>-</sup>) and a Fenton reaction — could be involved. Yet another possibility is a release of Ca<sup>2+</sup> from intracellular stores which could activate cellular nucleases and thus induce DNA damage (18,19).

The ratio of pyrimidine dimers and oxidative DNA modifications at various wavelengths depends on the relative contribution of direct and indirect mechanisms and is of major interest for the estimation of the mutagenic risk associated with solar irradiation and the impact of spectral changes caused by ozone layer depletion. Here, we report a quantification of cyclobutane pyrimidine dimers and various types of oxidative modifications induced in cultured cells by wavelengths between 290 and 500 nm. The lesions were quantified by means of several repair endonucleases (Table I) and an alkaline elution technique (28).

The results indicate that the generation of oxidative DNA base modifications by solar radiation has a second maximum between 400 and 450 nm due to the excitation of (unidentified) endogenous photosensitizers and that in cells exposed to sunlight these indirectly induced base modifications account for ~10% of the total endonuclease-sensitive base damage.

### Materials and methods

#### Cells and repair endonucleases

AS52 Chinese hamster ovary cells, which carry the bacterial *gpt* gene for analysis of mutations (29), were obtained from W.J.Casparly, Research Triangle

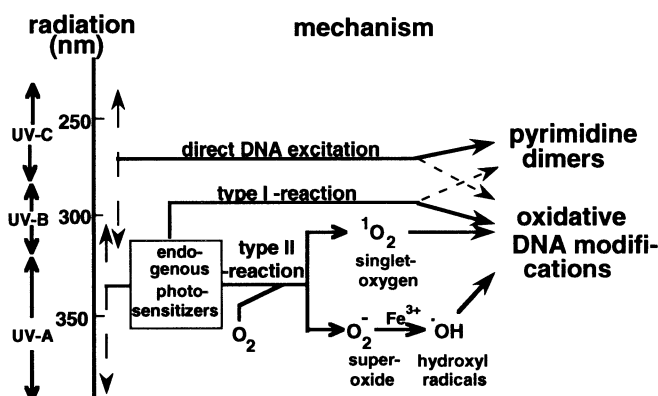


Fig. 1. Direct and indirect mechanisms for the induction of pyrimidine dimers and oxidative DNA modifications by UV and visible light.

**Table I.** Recognition of DNA modifications by repair endonucleases used in this study

Repair endonuclease	Recognition spectrum <sup>a</sup> Sites of base loss <sup>b</sup>	Base modifications
Fpg protein <sup>c</sup>	+	8-oxoG <sup>d</sup> ; Fapy <sup>e</sup>
Endonuclease III <sup>c</sup>	+	5,6-dihydropyrimidines; hyd <sup>f</sup>
T4 endonuclease V	+	Py <> Py <sup>g,h</sup>
Exonuclease III	+	—

<sup>a</sup>See refs 20–24.<sup>b</sup>For the recognition of sites of base loss oxidized in the 1' or 4' position, see ref. 25.<sup>c</sup>5-Hydroxycytosine and 5-hydroxyuracil have recently been described as new substrates of both Fpg protein and endonuclease III (26).<sup>d</sup>7,8-Dihydro-8-oxoguanine (8-hydroxyguanine).<sup>e</sup>Formamidopyrimidines (imidazole ring-opened purines).<sup>f</sup>5-Hydroxy-5-methylhydantoin and other ring-contracted and fragmented pyrimidines.<sup>g</sup>Cyclobutane pyrimidine photodimers.<sup>h</sup>At 100-fold higher concentration, 4,6-diamino-5-formamidopyrimidine (Fapy-A) was also found to be a substrate of T4 endonuclease V (27).

Park, USA, and cultured in Ham's F12 medium containing 5% fetal calf serum, penicillin (100 units/ml) and streptomycin (100 µg/ml).

Formamidopyrimidine-DNA glycosylase (Fpg protein) (30) and endonuclease III from *E.coli* were kindly provided by S.Boiteux, Fontenay aux Roses, France. T4 endonuclease V was partially purified by the method described by Nakabeppu *et al.* (31) from the *E.coli* strain A 32480 (*uvrA*, *recA*, F'lac IQ1). The strain carries the plasmid ptac-denV (kindly provided by L.Mullenders, Leiden, Netherlands). Expression of T4 endonuclease V is induced with isopropyl-β-D-thiogalactopyranoside. Exonuclease III was purchased from Boehringer Mannheim, Germany. All repair endonucleases were tested under cell-free conditions for their incision at reference modifications (i.e. thymine glycols induced by OsO<sub>4</sub>, AP sites by low pH and 8-oxoG by methylene blue plus light) to ensure that the correct substrate modifications are fully recognized and no incision at non-substrate modifications takes place (32).

#### Light sources and irradiation conditions

Irradiation with UV and visible light was carried out at 0°C in PBS buffer (137 mM NaCl, 2.7 mM KCl, 8.3 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, pH 7.4) either with a broad-spectrum UVB source (Philips TL20W/12RS; maximum emission at 306 nm) filtered by the polystyrene of the culture flasks (~1.3 W/m<sup>2</sup>), or with a 1000 W xenon arc lamp (976C-0010, Hanovia, USA) with a grid monochromator (bandwidth 8 nm) and/or cut-off filters (~4 W/m<sup>2</sup>/nm). For irradiation with the xenon lamp, the cells were suspended in a quartz tube (3×10<sup>6</sup> cells in 3 ml PBS buffer). The transmission of the cell suspension was >50% at all wavelengths between 290 and 600 nm. Cut-off filters (Schott, Mainz, Germany; 3 nm; two in line) with half-maximal absorptions at 335, 360, 400, 420, 455, 475 and 495 nm were used to define wavelength intervals in the determination of the action spectra. The dosimetry was performed with a calibrated dosimeter (Krochmann GmbH, Berlin, Germany) equipped with a GaP-photoelement.

#### Quantification of endonuclease-sensitive modifications

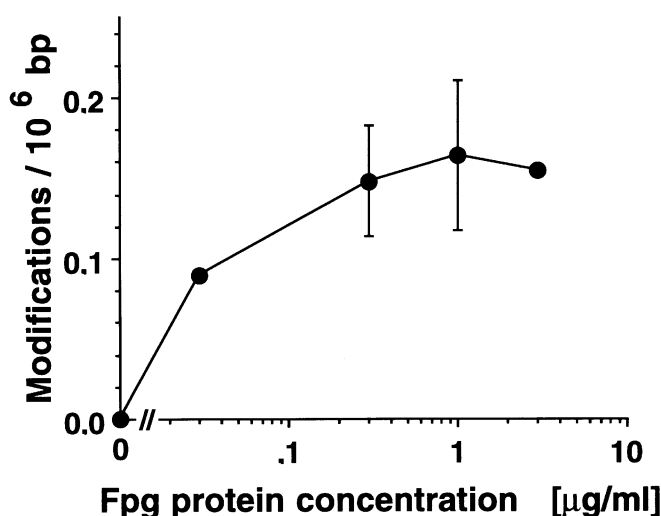
Determination of endonuclease-sensitive modifications was carried out by means of an alkaline elution assay, in which the cellular DNA was incubated with one of the repair endonucleases [Fpg protein (1 µg/ml), endonuclease III (10 ng/ml), T4 endonuclease V (30 ng/ml) or exonuclease III (0.5 µg/ml)] immediately after cell lysis, as described previously (28,33). To quantify DNA single-strand breaks, the incubation was carried out without endonuclease. The alkaline elution followed the method of Kohn *et al.* (34) with modifications (28,35). For the quantitative evaluation, the slope of an elution curve obtained with γ-irradiated cells was used for calibration (6 Gy = 1 ssb/10<sup>6</sup> bp). The slopes observed with untreated control cells were subtracted. The numbers of modifications incised by the repair endonucleases were obtained by subtraction of the number of single-strand breaks observed without endonuclease treatment.

#### Quantification of cyclobutane pyrimidine dimers by monoclonal antibodies

A monoclonal mouse H3 antibody (36) was used to quantify cyclobutane pyrimidine dimers in an immuno dot-blot assay as described previously (37). The antibody recognizes TT and 5'-TC cyclobutane dimers, which account for ~70% of all cyclobutane dimers induced by UVB (38), but not (6–4) photoproducts.

**Table II.** Comparison of quantifications of cyclobutane pyrimidine dimers by alkaline elution and immunoassay

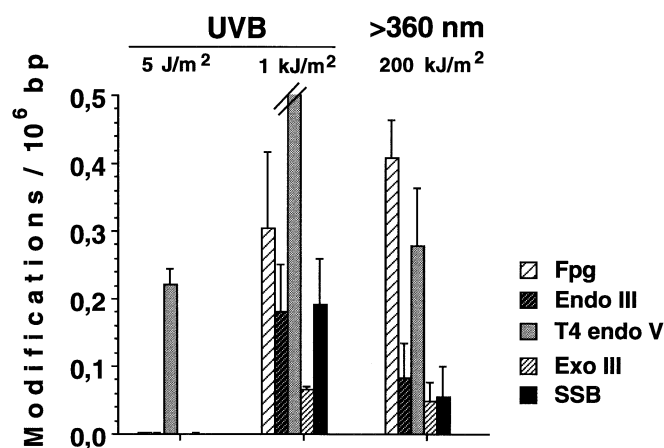
Inducing radiation	Dose applied (kJ/m <sup>2</sup> )		Modifications detected per kJ/m <sup>2</sup> per 10 <sup>6</sup> bp	
	Alk. elution	Immunoassay	Alk. elution <sup>a</sup>	Immunoassay <sup>b</sup>
300 nm	0.005	0.36–2.9	89±10	101±8
310 nm	0.2	17–34	3.7±0.5	3.2±0.2
320 nm	3	35–70	0.17±0.05	0.16±0.2
UVB	0.0066	0.79–2.4	77±13	139±39

<sup>a</sup>Sites sensitive to T4 endonuclease V determined by alkaline elution.<sup>b</sup>Sites detected in an immuno dot blot assay using the monoclonal mouse H3 antibody.**Fig. 2.** Recognition by various concentrations of Fpg protein of DNA modifications induced by visible light (>400 nm). Data are obtained from 1 or 3 independent experiments (± SD).

## Results

### Validation of the assay system

The reliability of the alkaline elution assay when used in combination with repair endonucleases to quantify endonuclease-sensitive DNA modifications was verified in three types of experiment. Firstly, cyclobutane pyrimidine dimers induced by UVB in AS52 (Chinese hamster ovary) cells were quantified both with T4 endonuclease V in combination with the alkaline elution technique and with an immunoassay (see Materials and methods). The two independent assay systems gave similar results (Table II). Secondly, the enzyme concentration dependence for the recognition by Fpg protein of visible light-induced oxidative modifications was determined. As shown in Figure 2, the number of sites detected by the enzyme was saturated at 1 µg/ml Fpg protein. This concentration is similar to that required for a full recognition of base modifications induced under cell-free conditions by methylene blue plus light (32). Thirdly, the recognition by the various enzymes of sites of base loss induced by methyl methane-sulfonate (MMS) was analysed. T4 endonuclease V, endonuclease III and exonuclease III detected the same number of modifications (data not shown), in agreement with the fact that sites of base loss are substrates for all three enzymes (Table I).



**Fig. 3.** DNA damage profiles induced in AS52 cells by broad spectrum UVB (Philips TL20W/12RS filtered by polystyrene culture flasks) and by wavelengths  $>360$  nm (xenon arc lamp). Columns indicate the numbers of various endonuclease-sensitive modifications (first to fourth column) and the number of single-strand breaks (fifth column). Data represent the means of 3–7 independent experiments ( $\pm$  SD).

#### Comparison of DNA damage profiles

The numbers of various endonuclease-sensitive modifications (see Table I) induced in AS52 by two spectral ranges of radiation are shown in Figure 3 in the form of DNA damage profiles. After broad spectrum UVB irradiation, 99% of all modifications detected were pyrimidine dimers sensitive to T4 endonuclease V. The sites sensitive to endonuclease III and Fpg protein were mostly base modifications, since the number of AP sites detected by exonuclease III was even lower. After irradiation with wavelengths above 360 nm from a xenon lamp, T4 endonuclease V-sensitive base modifications were still detectable (Figure 3). Since an additional cut-off filter at 360 nm had no effect on the number of modifications generated per dose unit (data not shown), the pyrimidine dimers cannot be attributed to low amounts of radiation emitted below 360 nm. Base modifications sensitive to Fpg protein were formed in a yield similar to pyrimidine dimers, while sites of base loss (sensitive to exonuclease III), single-strand breaks and oxidative pyrimidine modifications (sensitive to endonuclease III) were much less frequent.

#### Effects of cut-off filters on the damage profiles induced by UV radiation from a monochromator

Damage profiles observed in AS52 cells exposed to 310, 320 and 330 nm radiation from a monochromator (8 nm band width) are depicted in Figure 4. For all three wavelengths, the effects of one or two additional cut-off filters (with half-maximal absorption at 295, 305 and 320 nm, respectively) are also shown. The data indicate that the elimination of wavelengths well below the nominal settings of the monochromator had no effect on the generation of non-dimer modifications (single-strand breaks, modifications sensitive to endonuclease III and Fpg protein) and of pyrimidine dimers induced at 310 nm. However, the cut-off filters significantly reduced the number of pyrimidine dimers per dose unit induced with monochromator settings at 320 and 330 nm. A second cut-off filter of the same type had no further effect in any case. Therefore, a significant part—but not all—of the pyrimidine dimers induced at 320 nm and 330 nm can be attributed to the low percentage of radiation below 305 and 320 nm, respectively. In contrast, this low dose of radiation

with shorter wavelengths does not contribute significantly to the generation of the Fpg-sensitive modifications. Therefore, different mechanisms must be responsible for the two types of damage.

#### Dose dependence

The number of pyrimidine dimers sensitive to T4 endonuclease V increased linearly with dose in the dose range analysed (i.e., in which 0.05–1 modifications per  $10^6$  bp were induced). In contrast, the number of Fpg-sensitive modifications induced in the visible range of the spectrum followed a saturation curve, i.e. the extent of damage did not exceed 0.3 modifications per  $10^6$  bp (Figure 5).

#### Action spectra

The generation of single-strand breaks and modifications sensitive to T4 endonuclease V and Fpg protein in AS52 cells at various wavelengths calculated for a given dose ( $20 \text{ kJ/m}^2$ ) is shown in Figure 6 in the form of action spectra. In all experiments, the actual doses applied were chosen to yield  $<1$  modification per  $10^6$  bp. Up to the doses applied, all types of modifications were shown to increase linearly with dose (see above). A monochromator in line with an additional cut-off filter (with half-maximal absorption at wavelengths 10–15 nm below the nominal settings of the monochromator) was used in the range between 290 and 330 nm. For longer wavelengths, no monochromator could be used and the differences observed in experiments with cut-off filters (two in line) at two different wavelengths were determined. These were used to calculate the number of modifications per unit dose for the arithmetic mean of the two cut-off wavelengths.

The action spectra indicate that numbers of single-strand breaks, Fpg-sensitive modifications and T4 endonuclease-sensitive sites (cyclobutane pyrimidine dimers) decreased in parallel between 290 and  $\sim 315$  nm. The T4 endonuclease-sensitive sites continued to decrease exponentially at longer wavelengths, but were still detectable up to 380 nm. In contrast, the number of Fpg-sensitive modifications per unit dose had a minimum around 340 nm and rose to a long-wave maximum between 400 and 450 nm. Single-strand breaks were detectable in all wavelength ranges. The yield was similar to that of Fpg-sensitive modifications in the UVB range, but was much lower than that of Fpg-sensitive modifications in the visible range.

#### Discussion

The results presented above provide quantitative information on the ratio of pyrimidine dimers and oxidative DNA base modifications induced in mammalian cells at various wavelengths of solar radiation. In addition, they allow mechanistic conclusions and an estimation of the contribution of oxidative base modification to the mutagenicity of sunlight.

The wavelength dependence of the induction of pyrimidine dimers (T4 endonuclease sensitive sites) in AS52 cells (Figure 6) closely resembles that reported previously for other types of cultured cells (15,39). It is also similar to that observed for the induction of both cyclobutane pyrimidine dimers and (6–4) photodimers in isolated DNA (40). The induction of dimers in the UVA range of the spectrum therefore can be attributed to the (very weak) end absorption of DNA and need not be mediated by other cellular chromophores, which in principle could generate cyclobutane pyrimidine dimers by triplet-triplet energy transfer (33,41). The recently reported (27) weak recognition by T4 endonuclease V of 4,6-diamino-

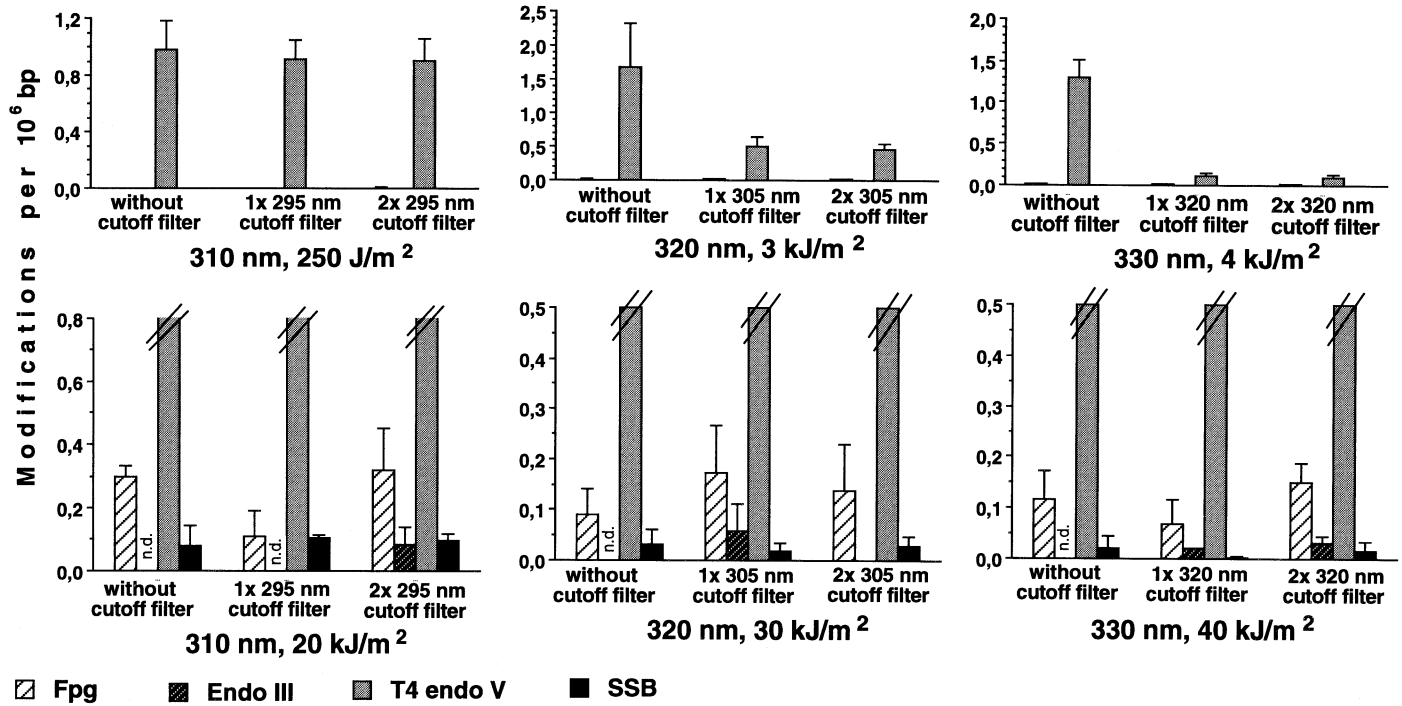


Fig. 4. Effects of one or two cut-off filters on the DNA damage profiles induced in AS52 cells by radiation from a monochromator-coupled xenon lamp (band width 8 nm). Data represent the means of 3–6 independent experiments ( $\pm$  SD).

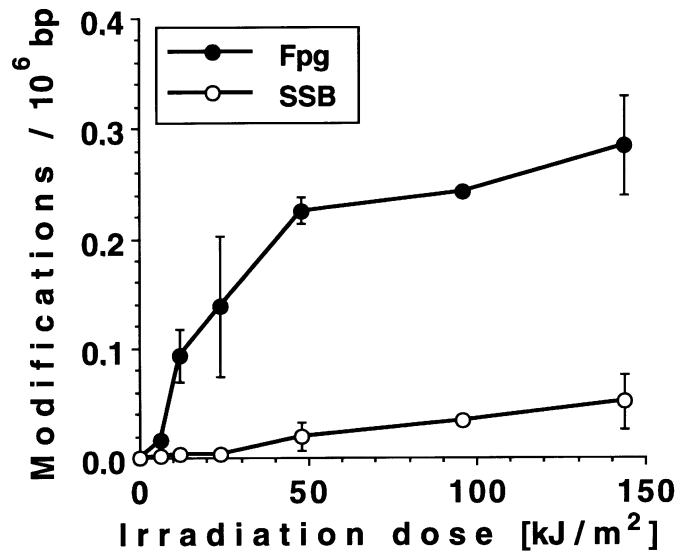


Fig. 5. Dose dependence of the induction of Fpg-sensitive modifications (●) and single-strand breaks (○) in AS52 cells by visible light (>400 nm) from a xenon arc lamp. Dose values given on the abscissa refer to the range between 400 and 500 nm. Data represent the means of 3–5 independent experiments ( $\pm$  SD).

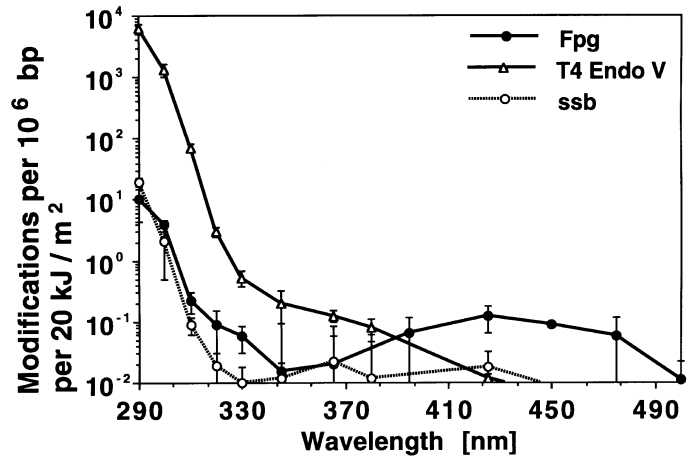


Fig. 6. Wavelength dependence of the induction of Fpg-sensitive modifications (●) T4 endonuclease V-sensitive modifications ( $\Delta$ ) and single-strand breaks (○) in AS52 cells calculated for a dose of 20 kJ/m<sup>2</sup>. Between 290–330 nm, radiation passed through a grid monochromator (band width 8 nm) and an additional cut-off filter with half-maximal absorption 5–15 nm below the nominal wavelength of the monochromator. Between 340 and 500 nm, only cut-off filters were used (see description in the text). In the dose range applied for the determinations, all types of modifications were shown to increase linear with dose. Data represent the means of 3–7 independent experiments ( $\pm$  SD).

5-formamidopyrimidine (Fapy-A) cannot account for the T4 endonuclease-sensitive sites induced by UVA since (i) the enzyme concentrations required to for a (very incomplete) excision of Fapy-A were >300-fold higher than those used in this study and (ii) Fapy-A is a good substrate for Fpg protein and there is only a narrow wavelength range (around 380 nm) in which there is a significant recognition by T4 endonuclease V in the presence of an (at least) similarly high number of Fpg-sensitive sites.

In contrast to pyrimidine dimers, base modifications

sensitive to Fpg protein are induced in the whole wavelength range between 290 and 470 nm (Figure 6). Within the UVB range (290–315 nm), the ratio of Fpg-sensitive modifications and pyrimidine dimers is nearly constant and follows the DNA photon absorption. Therefore, these Fpg-sensitive modifications arise (mostly) from the direct excitation of DNA. Chemically, they may consist of both 8-oxoG and formamidopyrimidines. Both types of modification are established substrates of Fpg protein (Table I). 8-OxoG has

been detected in UVB-irradiated mammalian cells (42). Formamidopyrimidines have been identified in DNA irradiated under cell-free conditions with UVB (43). Since according to a recent report, 5-hydroxycytosine and 5-hydroxyuracil are additional substrates of both Fpg protein and endonuclease III (26), the presence of these lesions (up to the extent of endonuclease III-sensitive base modifications) can also not be excluded. Only some of the Fpg-sensitive modifications are sites of base loss, since the number of sites detected by exonuclease III, which recognizes sites of base loss only (Table I), is much lower.

Beyond 350 nm, the yield of Fpg-sensitive base modifications increases to a relative maximum between 400 and 450 nm (Figure 6). In this range of the spectrum, no or very few other modifications (single-strand breaks, AP sites, pyrimidine modifications sensitive to endonuclease III) are generated (Figures 3 and 6), as observed previously for another cell line (44). The damage profile thereby resembles that induced by singlet oxygen or type I photosensitizers under cell-free conditions (35), in support of the assumption that endogenous photosensitizers are responsible and modify the DNA either directly (type I reaction) or via singlet oxygen (type II reaction), but not via hydroxyl radicals. Since according to cell-free studies (45), only a few formamidopyrimidines are generated by these mechanisms, 8-oxoG is likely to be the principal modification produced in this wavelength range. The formation of 8-oxoG in cells treated with riboflavin and visible light has been reported (46).

Like Fpg-sensitive modifications, single-strand breaks were induced in all ranges of the spectrum, in agreement with earlier observations that they too can be generated by both direct and indirect mechanisms (11). In addition, they may (in part) arise from sites of base loss, in particular oxidized sites of base loss, which are converted into strand breaks under the alkaline elution conditions.

The generation of base modifications sensitive to endonuclease III is largely restricted to UVB (Figure 3). According to earlier studies, these endonuclease III-sensitive modifications are cytosine hydrates and other oxidized pyrimidines formed in consequence of a direct excitation of DNA in a yield of ~1% of that of cyclobutane pyrimidine dimers (47–49).

The action spectra shown in Figure 6 in combination with published solar emission spectra (50,51) make it possible to calculate that solar radiation induces Fpg-sensitive base modifications, most of which presumably are 8-oxoG, in a yield of up to 10% of that of pyrimidine dimers. Exposures of keratinocytes for a few minutes to direct sunlight indeed confirmed that Fpg-sensitive modifications and T4 endonuclease-V-sensitive modifications are generated in a ratio of 1:10 (data not shown). At the *gpt* locus of AS52 cells, the number of Fpg-sensitive modifications induced by various (exogenous) photosensitizers that gave rise to (or at least was associated with) a given number of mutations was similar to the number of T4 endonuclease-V-sensitive modifications that resulted in the same mutation frequency (unpublished results). Therefore, oxidative base modifications are calculated to be responsible for several percent of the overall mutagenicity of sunlight. Indeed, sequence analysis of mutations induced by simulated sunlight and UVA in cultured cells revealed a high proportion of T→G transversions, which are not observed with UVC (52). However, this type of base substitution is different from that known to result from 8-oxoG formation. The early saturation observed for the induction of Fpg-sensitive modi-

fications by UVA and visible light (Figure 5) indicates that both the number of oxidative DNA modifications and their mutagenic (or carcinogenic) consequences can be underestimated when experiments with high doses and dose rates are extrapolated to the low doses and dose rates which may be relevant for many cells in the human skin.

## Acknowledgements

This work was supported by the Bundesministerium für Bildung und Forschung (07UVB51) and by the European Commission (EV5V-CT-94-0458). We thank S.Boiteux for providing Fpg protein and endonuclease III.

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Received on October 2, 1996; revised and accepted on November 26, 1996