8-Hydroxydeoxyguanosine Formation at the 5' Site of 5'-GG-3' Sequences in Double-stranded DNA by UV Radiation with Riboflavin*

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DNA damage caused by UV radiation in the presence of riboflavin or hematoporphyrin was characterized by the DNA sequencing technique using 32P-labeled DNA fragments and the analysis of 8-hydroxydeoxyguanosine (8-OH-dG) formation in calf thymus DNA. Exposure of double-stranded DNA to 345 or 302 nm radiation in the presence of riboflavin induced the sequence-specific DNA cleavage which is different from that caused by 302 or 254 nm irradiation in the absence of a sensitizer. The specific cleavage sites were the guanine residues located 5' to guanine. On the other hand, when denatured single-stranded DNA was irradiated at 365 nm with riboflavin or hematoporphyrin, cleavages occurred at most guanine residues. With D2O, the sequence-specific damage of double-stranded DNA by riboflavin was not enhanced, whereas the damage to single-stranded DNA by hematoporphyrin was greatly enhanced. Photodynamic action of riboflavin caused the formation of 8-OH-dG in double-stranded DNA. The enhancing effect of D2O on 8-OH-dG formation was not observed with riboflavin. By contrast, hematoporphyrin plus 365-nm light induced the 8-OH-dG formation only in denatured single-stranded DNA and the 8-OH-dG yield was increased about 2-fold in D2O. ESR spin destruction experiments suggested that photoexcited riboflavin reacts with DgMP to produce riboflavin adrenalin radical or 30-amine cation radical, but not with other mononucleotides. The estimated ratio of 8-OH-dG yield to total guanine loss indicates that the photoexcited riboflavin induces 8-OH-dG formation specifically at the guanine residue located 5' to guanine through electron transfer. The mechanism was discussed in relation to UV carcinogenesis.

Sunlight-induced skin cancers are thought to result from DNA damage produced by radiation in the UV region (1, 2). The predominant base modifications formed by irradiation at wavelength below 280 nm are the cyclobutane pyrimidine dimers and the pyrimidine (6-4) photoproducts (3-5). A number of studies have suggested that there is a causal relationship between the induction of the pyrimidine photoproducts and UV carcinogenesis (6). However, whereas the action spectra for the formation of dimers and (6-4) photoproducts decline sharply with increasing wavelengths above 280 nm, the action spectra of mutation induction and tumorigenesis show a prominent shoulder above 300 nm (2, 7). Wavelengths in the UV-A (320-380 nm) region have been shown to cause skin cancer in animals when given in high doses over a long period of time (8, 9). The UV-A wavelengths were reported to induce mutation and cellular transformation (10). Therefore, it is possible that DNA lesions other than the pyrimidine photoproducts are responsible for the mutagenic and carcinogenic effects of UV-A and possibly even UV-B (290-320 nm).

Since DNA does not absorb UV-A, endogenous non-DNA chromophores appear to be responsible for the UV-A-induced DNA damage. There are abundant porphyrin, flavin, and other cellular molecules that could act as photosensitizers. Indeed, various endogenous photosensitizers have been shown to enhance the DNA damage induced by UV-A (10). However, the mechanisms and products of the photosensitized DNA damage were not well understood. Two major types of reaction are well established for the photosensitization. Type I involves the direct interaction of the sensitizer, usually in triplet state, with the substrate through electron transfer reactions and Type II involves energy transfer from the triplet state of sensitizer to oxygen, resulting in the formation of singlet oxygen. Our previous paper demonstrated that photodynamic guanine modification of single-stranded DNA by hematoporphyrin is mediated by singlet oxygen (11).

8-Hydroxydeoxyguanosine (8-OH-dG) is considered to be one of the oxidative DNA products induced by oxygen radicals (12). Singlet oxygen has been reported to cause the formation of 8-OH-dG in DNA (13). Relevantly, Floyd et al. (14) have reported that methylene blue plus light caused 8-OH-dG formation in DNA with singlet oxygen as a mediator. It has drawn much interest that the formation of 8-OH-dG causes misreplication of DNA that might lead to mutation or cancer (15). It is now very important to study the mechanism by which UV-A and UV-B induce site-specific DNA damage and 8-OH-dG formation in the presence of an endogenous sensitizer.

This paper describes studies on riboflavin (as a model of endogenous compound)-sensitized photodamage of DNA by using 32P 5'-end-labeled DNA fragments obtained from human c-Ha-ras-1 protooncogene. We also investigated the photodynamic 8-OH-dG formation with riboflavin in calf thymus DNA by an electrochemical detector coupled to an HPLC (HPLC-ECD), and the reaction mechanism by an ESR.

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The abbreviations used are: 8-OH-dG, 8-hydroxy-2'-deoxyguanosine (and also known as 8-oxo-7,8-dihydro-2'-deoxyguanosine); DTPA, diethylenetriaminepentaacetic acid; HPLC, high pressure liquid chromatography; HPLC-ECD, electrochemical detector coupled to an HPLC; 4-oxo-TEMPO, 2,2,6,6-tetramethyl-4-piperidine-N-oxyl; W, watt(s).
spin destruction method. The results were compared with those of another sensitizer (hematoporphyrin).

**EXPERIMENTAL PROCEDURES**

**Materials**—[α-32P]ATP (6000 Ci/mmole) was supplied by Du Pont-New England Nuclear. Calf intestine alkaline phosphatase (3000 units/mg) was obtained from Boehringer Mannheim GmbH, Mannheim, Germany. Riboflavin and hematoporphyrin dihydrochloride were purchased from Nacalai Tesque Co., Kyoto, Japan. 4-Oxo-TEMPO was purchased from Aldrich. DTPA was from Dojin Chemicals Co., Kumamoto, Japan. Calf thymus DNA was from Sigma. Nuclease P1 (400 units/mg) was from Yamasa Shoyu Co., Chiba, Japan. D2O (99.95%) was obtained from Commissariat à l’Energie Atomique in France.

**Preparation of 32P 5'-end-labeled DNA Fragments**—DNA fragments were prepared from plasmid pbcNI, which carries a 6.6-kilobase BamHI chromosomal DNA segment containing human c-Ha-ras-1 protooncogene (16). A singly labeled 261-base pair fragment (AvaI*1645 to XbaI*2105), 341-base pair fragment (XbaI*2106 to AvaI*2246), and 337-base pair fragment (PstI*2345 to AvaI*2681) were obtained as previously described (16). The asterisk indicates 32P labeling, and nucleotide numbering starts with BamHI site (17).

**UV Radiation of DNA in the Presence of Sensitizer**—The standard reaction mixture in a microtube (1.5-ml Eppendorf) contained 0.05 mM riboflavin or 0.1 mM hematoporphyrin, ~0.1 μM/base [32P]DNA fragment (~0.27 Ci/mmol), and 2 μM/base sonicated calf thymus DNA (100,000 ρg/ml) of 10 mM sodium phosphate buffer at pH 7.9 containing 5 μM DTPA. The reaction mixture was usually irradiated with five 8-W UV lamps (302 or 365 μm, UVP, Inc., San Gabriel, CA) placed at a distance of 15 cm. UV doses were measured using a UVX radiometer (UVP). The reaction mixture was protected from direct sunlight, and the temperature was maintained at 0 °C. After irradiation, the DNA fragments were heated at 90 °C in 1 M piperidine for 20 min where indicated and treated as previously described (16, 18).

Denatured DNA fragments were prepared by heating double-stranded DNA fragments at 90 °C for 5 min and quick chilling. A single-stranded 40-base-long DNA fragment (5′-GTCGACCTTTCTCTT-CTTTCCTTGGCGGAATCGTTACGTAC-3′) and the complementary strand were synthesized by an Applied Biosystems model 381A DNA synthesizer (Applied Biosystems, Foster City, CA). Where indicated, D2O was used instead of H2O. The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam-Gilbert procedure (18) using a DNA sequencing system (LKB2010 Macrophor). A laser densitometer (LKB 2222 UltraScan XL) was used for the measurement of the relative amounts of oligonucleotides from treated DNA fragments.

**Analysis of 8-OH-dG in Calf Thymus DNA Formed by 365 nm Irradiation with Sensitizer**—[5′-32P]ATP was measured by a modified method of Kasai et al. (19). Calf thymus DNA (50 μM/base) was irradiated with UV-A (365 mm, 40 W) in the presence of 0.1 mM riboflavin or 0.1 mM hematoporphyrin in 100 μl of 4 mM phosphate buffer (pH 7.9) at 0 °C. After irradiation, the DNA was separated by ethanol precipitation, dissolved in 20 mM acetate buffer (pH 5.0) and digested to deoxyribonucleosides by incubation first with 8 units of nuclease P1 at 37 °C for 30 min and then with 1.3 units of calf intestine alkaline phosphatase at 37 °C for 1 h in 0.1 M Tris-HCl buffer (pH 7.5). The resulting deoxyribonucleosides mixture was injected into an HPLC apparatus (LC-6A, Shimadzu, Kyoto, Japan) equipped with both a UV detector (SPD-6A, Shimadzu) and an electrochemical detector (coulochem model 5200-2, ESA, Bedford, MA). Chromatography was performed on Beckman Ultrasphere ODS (0.46 × 25 cm); eluent, 10% aqueous methanol containing 12.5 mM citric acid, 25 mM sodium acetate, 30 mM NaOH, and 10 mM acetic acid; flow rate 1 ml/min. The molar ratio of 8-OH-dG to deoxyguanosine (dG) in each DNA sample was measured based on the peak height of authentic 8-OH-dG with the electrochemical detector and the UV absorbance at 254 nm of dG.

**Detection of Riboflavin Radical by ESR Spin Destruction Method**—Radicals of hematoporphyrin and riboflavin were measured by the modified Moan’s method using 4-oxo-TEMPO (20, 21). Typical samples (50 μl) contained 29 mM sodium phosphate buffer (pH 7.9), 0.1 mM riboflavin or 0.2 mM hematoporphyrin, and 5 μM 4-oxo-TEMPO. Samples were irradiated with UV-A (365 nm, 40 W) placed at 15 cm in a microtube (1.5-ml Eppendorf) in equilibrium with the atmosphere at 0 °C. For ESR measurements, aliquots of the solutions were taken in calibrated capillaries (Clay Adams, 50 μl) before irradiation or after different irradiation times. ESR spectra were measured at room temperature using a JES-Fe-3XG (JEOL, Tokyo, Japan) spectrometer with 100-KHz field modulation according to the previously described method (11). The magnetic fields were calculated by the splitting of Mn2+ in MgO (ΔHSE = 86.9 G). Spectra were recorded with a microwave power of 1 mW and a modulation amplitude of 1.0 G.

**RESULTS**

**DNA Cleavage by 365 nm Radiation in the Presence of Riboflavin or Hematoporphyrin**—[5′-end-labeled DNA fragments were exposed to 365 nm radiation in the presence of riboflavin or hematoporphyrin, and the extent of DNA damage was estimated by gel electrophoretic analysis. Fig. 1A shows the autoradiogram of double-stranded DNA fragments treated with riboflavin plus 365-nm light. The upper and lower bands in the control show single-stranded and double-stranded forms of intact DNA fragments, respectively. Photodegradation of DNA with riboflavin increased with irradiation dose. The DNA cleavage was enhanced approximately 10-fold upon piperidine treatment (Fig. 2A, lane 2 and 3). Since piperidine breaks the DNA at a sugar with a modified base or a sugar without a base (18), it is suggested that base alteration and/or liberation occurred with exposure to 365-nm light in the presence of riboflavin. Exposure of double-stranded DNA to 365 nm radiation with hematoporphyrin produced no photodamage (Fig. 2B, lane 3). However, when the double-stranded DNA fragments were denatured to single-stranded DNA by heating at 90 °C for 5 min, 365 nm radiation with hematoporphyrin induced DNA damage (Fig 1B). No

2We think that a band of double-stranded DNA in the denatured DNA sample (Fig. 1B, lane 1), which appears to disappear with increased UV dose, probably resulted from renaturation of the denatured DNA after piperidine treatment; it is likely that if the denatured DNA is not cleaved, the renaturation will easily occur, but with increased cleavage of single-stranded DNA, the renaturation will less likely occur, resulting in the disappearance of the band corresponding to double-stranded DNA.
oligonucleotides was produced without a sensitizer (Fig. 1, lane 6) or without UV radiation (Fig. 1, lane 1). In addition, experiments using a synthetic single-stranded DNA fragment (40 bases) and the corresponding complementary duplex demonstrated that hematoporphyrin plus 365-nm light caused cleavage of single-stranded DNA but not cleavage of double-stranded DNA (data not shown). These results were consistent with our previous findings that DNA damage by hematoporphyrin plus white light is specific for single-stranded DNA (11). Therefore, although there remains a possibility that partially double-stranded form of DNA might exist in the denatured preparation, the damage observed with denatured DNA in the presence of hematoporphyrin is regarded as the damage of single-stranded DNA, and we hereafter refer to the denatured DNA as the denatured single-stranded DNA.

Effect of D2O on DNA Damage by 365 nm Radiation with Riboflavin or Hematoporphyrin—The usual test for the participation of singlet oxygen in a reaction is to observe the effect of D2O, in which the lifetime of singlet oxygen is 10 times or more longer than in H2O (22). Fig. 2B shows effect of D2O on DNA damage by 365 nm radiation in the presence of riboflavin or hematoporphyrin. Enhancement of DNA damage in D2O was observed with hematoporphyrin (lanes 1 and 2). D2O effect was not observed with riboflavin-mediated damage to double-stranded DNA (lanes 4 and 5). These results suggest that damage of single-stranded DNA by 365 nm irradiation with hematoporphyrin is due to photochemically generated singlet oxygen, whereas riboflavin-sensitized degradation of double-stranded DNA is not due to singlet oxygen.

Site Specificity of DNA Cleavage by UV Radiation with Riboflavin or Hematoporphyrin—To examine the sites of DNA cleavage, 32P 5' end-labeled DNA fragments, treated with 365 or 302 nm radiation in the presence of riboflavin or hematoporphyrin and subsequently with piperidine, were electrophoresed and the autoradiogram was scanned with a laser densitometer (Figs. 3-5). The cleavage sites were determined by utilizing the Maxam-Gilbert procedure (18). Fig. 3 demonstrates that 365 nm irradiation of double-stranded DNA in the presence of riboflavin induced cleavages specifically at the 5' site of 5'-GGG-3' sequences. The most preferred site of 5'-GGG-3' was the central guanine. Although the cleavages at the positions of guanine located 5' to adenine occurred slightly, no or few cleavages were observed at other sequences, including a sequence in which guanine is located 5' to a pyrimidine nucleoside. On the other hand, when denatured single-stranded DNA was irradiated at 365 nm in the presence of riboflavin or hematoporphyrin, the cleavage occurred at most guanine residues (Fig. 4). Exposure of double-stranded DNA to 302 nm radiation with riboflavin induced sequence-specific DNA cleavage similar to that obtained with 365 nm radiation. **Fig. 2. Effects of piperidine and D2O on riboflavin or hematoporphyrin-mediated photodegradation of DNA.** A, the 32P 5'-end-labeled 337-base pair fragment (PstI 2345 to AuaI 2681) was exposed to 3.3 J/cm² UV light (365 nm) with 0.05 mM riboflavin in 100 µl of 10 mM sodium phosphate buffer (pH 7.9) containing 2 µM/base sonicated calf thymus DNA and 5 µM DTPA. After irradiation, the DNA fragments were untreated (lane 2) or treated with 1 mM piperidine at 90 °C for 20 min (lane 3) prior to electrophoresis on an 8 M urea-containing 8% polyacrylamide gel. Lane 1 shows the electrophoresis pattern of untreated control. B, the fragment was exposed to 3.3 J/cm² UV light (365 nm) with riboflavin or hematoporphyrin under the conditions described in A. The DNA fragments were treated with piperidine prior to electrophoresis. Where indicated, D2O was used in place of H2O. For the experiment with denatured DNA, the 5'-end-labeled DNA fragment was treated at 90 °C for 5 min and quickly chilled. Lane 1, 0.1 mM hematoporphyrin, denatured DNA; lane 2, 0.1 mM hematoporphyrin, denatured DNA, D2O; lane 3, 0.1 mM hematoporphyrin, double-stranded DNA; lane 4, 0.05 mM riboflavin, double-stranded DNA; lane 5, 0.05 mM riboflavin, double-stranded DNA, D2O.

**Fig. 3. Site specificity of DNA cleavage induced by 365 nm radiation in the presence of riboflavin.** A, the 32P 5'-end-labeled 341-base pair fragment (XbaI 1906 to AuaI 2240) was exposed to 6.6 J/cm² UV light (365 nm) with 0.05 mM riboflavin in 100 µl of 10 mM sodium phosphate buffer (pH 7.9) containing 2 µM/base sonicated calf thymus DNA and 5 µM DTPA. B, the 32P 5'-end-labeled 261-base pair fragment (AucI 1645 to XbaI 1905) was used. After piperidine treatment, the DNA fragments were electrophoresed on an 8 M urea-containing 8% polyacrylamide gel using a DNA-sequencing system and the autoradiogram was obtained by exposing x-ray film to the gel. The relative amounts of oligonucleotides produced were measured by scanning the autoradiogram with a laser densitometer. The DNA fragments were degraded approximately 75% in A and 90% in B. The piperidine-labile sites of the treated DNA were determined by direct comparison of the positions with those produced by the chemical reactions of the Maxam-Gilbert procedure (18). **Horizontal axis**, the nucleotide number of human c-Ha-ras-1 protooncogene starting with the BamHI site (17).
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FIG. 4. Comparison of photoreactivity of DNA with riboflavin and hematoporphyrin. The 32P 5'-end-labeled 337-base pair fragment (PstI2345 to AvaI*2681) was exposed to 6.6 J/cm² UV light (365 nm) with 0.1 mM hematoporphyrin (A) or 0.05 mM riboflavin (B and C) in 100 µl of 10 mM sodium phosphate buffer (pH 7.9) containing 2 µM/base sonicated calf thymus DNA and 5 µM DTPA. For the experiment with denatured DNA (A and C), the 5'-end-labeled DNA fragment was treated at 90 °C for 5 min and quickly chilled before the addition of a sensitizer. After the piperidine treatment, the DNA fragments were analyzed by the method described in legend to Fig. 3. The DNA fragments were degraded approximately 90% in A, 95% in B, and 80% in C. Horizontal axis, the nucleotide number of human c-Ha-ras-1 protooncogene starting with the BamHI site (17).

radiation with riboflavin. In contrast, a high dose of 302 nm radiation in the absence of a sensitizer induced piperidine-labile lesions frequently at positions of cytosine located 3' to a pyrimidine base, although not all cytosine residues located 3' to pyrimidines were cleavage sites (Fig. 5). Similarly, exposure of DNA to 254 nm radiation in the absence of a sensitizer resulted in cleavages frequently at the sequences 5'-TC-3' and 5'-CC-3', as previously reported (23).

Formation of 8-OH-dG in Calf Thymus DNA by UV Radiation with Riboflavin or Hematoporphyrin—By using HPLC-ECD, we measured 8-OH-dG content in calf thymus DNA exposed to 365 nm radiation in the presence of riboflavin or hematoporphyrin. Riboflavin plus light increased 8-OH-dG content in both double-stranded and denatured single-stranded DNA with increasing the radiation dose, but the amount of 8-OH-dG produced in the double-stranded DNA was about 4 times more than that produced in the denatured DNA.

FIG. 5. Site specificity of DNA cleavage induced by 302 nm radiation in the presence or absence of riboflavin. The 32P 5'-end-labeled 337-base pair fragment (PstI2345 to AvaI*2681) was exposed to 0.5 J/cm² UV light (302 nm) with 0.1 mM riboflavin (A) or 5 J/cm² without a sensitizer (B) in 100 µl of 10 mM sodium phosphate buffer (pH 7.9) containing 2 µM/base sonicated calf thymus DNA and 5 µM DTPA. After piperidine treatment, the DNA fragments were analyzed by the method described in legend to Fig. 3. The DNA fragments were degraded approximately 10% in A and 10% in B. Horizontal axis, the nucleotide number of human c-Ha-ras-1 protooncogene starting with the BamHI site (17).

FIG. 6. 8-OH-dG formation in DNA irradiated with 365-nm light in the presence of riboflavin or hematoporphyrin. Calf thymus DNA (50 µM/base) was exposed to 365 nm radiation in the presence of 0.1 mM riboflavin (A; ○, Δ) or 0.1 mM hematoporphyrin (B; ▲) in 100 µl of 4 mM sodium phosphate buffer (pH 7.9) at 0 °C. Where indicated, D₂O was used in place of H₂O (closed symbols). For the experiment with denatured DNA (△, ▼, ◄), the 5'-end-labeled DNA fragment was treated at 90 °C for 5 min and quickly chilled before the addition of a sensitizer. After irradiation, the DNA was subjected to enzyme digestion and analyzed by HPLC-ECD as described under "Experimental Procedures."
DNA (Fig. 6A). On the other hand, photodynamic action of hematoporphyrin caused 8-OH-dG formation in denatured single-stranded DNA (Fig. 6B) but not in double-stranded DNA (data not shown). Interestingly, formation of 8-OH-dG by riboflavin was not enhanced in D₂O in both double-stranded and denatured single-stranded DNA (Fig. 6A). The results suggest that singlet oxygen plays little role in the riboflavin-sensitized 8-OH-dG formation in DNA. In contrast, the 8-OH-dG formation by hematoporphyrin plus 365-nm light was increased about 2-fold in D₂O, indicating the involvement of singlet oxygen (Fig. 6B).

**Activity of Photoexcited Riboflavin with dGMP**—The reactivity of photoexcited riboflavin with mononucleotides was investigated by the spin destruction experiment with 4-oxo-TEMPO, which is a stable nitroxide radical. Fig. 7 shows that marked loss of the nitroxide radical occurred during 365 nm irradiation of riboflavin in the presence of dGMP, whereas the decrease of nitroxide radical did not occur in the absence of dGMP. The addition of dAMP, dCMP, or dTMP caused only a small decrease of the radical (data not shown). The result suggests that photoexcited riboflavin preferentially reacts with dGMP to produce riboflavin radical, which destructs the nitroxide radical. During the irradiation of hematoporphyrin, a loss of the nitroxide radical was observed, as previously reported (20). However, the addition of dGMP had no effect on the rate of loss of nitroxide, suggesting no reactivity of hematoporphyrin radical with dGMP. Similar results were obtained by our previous experiments with white light (11).

**Discussion**

The present results using the DNA sequencing technique demonstrated that exposure of DNA to 302 nm radiation produced piperidine-labile photoproducts at positions of cytosine and, much less frequently, thymine located 3' to a pyrimidine base. Similar site-specific DNA cleavage was observed with 254 nm radiation even at doses much lower than those required with 302 nm radiation. The data with 254 nm radiation support the previous findings that pyrimidine (6-4) photoproducts occur predominantly at 5'-TC-3' and 5'-CC-3' sequences (4, 23). However, when a photosensitizer was added, even a low dose of 302 nm radiation gave DNA cleavage pattern different from that produced with pyrimidine (6-4) photoproducts. Exposure of double-stranded DNA to 365 or 302 nm radiation in the presence of riboflavin and subsequent piperidine treatment resulted in specific cleavages at the 5' site of 5'-GG-3' sequences. The most preferred site of 5'-GGG-3' was the central guanine. When denatured single-stranded DNA was used, hematoporphyrin as well as riboflavin induced alteration of most guanine residues. Although the site-specific cleavages at the sequence 5'-GG-3' were also observed to some extent with the denatured DNA exposed to 365 nm radiation plus riboflavin, there remains a possibility that the cleavage occurred in the double-stranded form which resided in the denatured DNA preparation. Riboflavin has been shown to interact specifically with deoxyguanosine upon irradiation of 450-nm light (24) and to enhance DNA breakage caused by 334 nm UV radiation (25, 26). Under the conditions we employed, the enhancing effect of D₂O on denatured single-stranded DNA damage was observed to a large extent with hematoporphyrin, suggesting the involvement of singlet oxygen. On the other hand, the enhancing effect of D₂O on double-stranded DNA was not observed with riboflavin. It is, therefore, considered that singlet oxygen plays only a little role, if any, in riboflavin-sensitized site-specific degradation of double-stranded DNA.

Floyd et al. (27) reported that riboflavin in combination with white light did not cause formation of 8-OH-dG in DNA, although methylene blue plus white light caused 8-OH-dG formation in DNA with singlet oxygen as a possible mediator (12). However, the present study shows that 365 nm irradiation of calf thymus DNA with riboflavin caused the formation of 8-OH-dG in both double-stranded and denatured single-stranded DNA. The 8-OH-dG formation was not enhanced in D₂O with either the double-stranded or the denatured DNA. On the other hand, photodynamic action by hematoporphyrin caused the formation of 8-OH-dG in denatured single-stranded DNA and the 8-OH-dG formation was enhanced about 2-fold in D₂O. These results indicate that 8-OH-dG formation of double-stranded DNA with riboflavin proceeds through the direct interaction of the photoexcited riboflavin with DNA. The ratios of the 8-OH-dG formation to total guanine loss estimated from the data with various UV doses (shown in Fig. 6A) and the data with various concentrations of riboflavin (data not shown) were approximately from 20 to 70% under the conditions employed; the ratio was decreased as the UV dose or the riboflavin concentration was increased. In addition, recent studies have shown that piperidine treatment of 8-OH-dG-containing DNA results in breakage of deoxyribose phosphate backbone (28). It is, therefore, considered that when riboflavin is interacted with consecutive guanine residues of double-stranded DNA and irradiated, the photoexcited riboflavin induces 8-OH-dG formation specifically at the guanine residue located 5' to guanine.

Mooman developed a new method to determine indirectly the amount of porphyrin radical in aqueous solutions (20). The method is based on the reaction of porphyrin anion radicals with 4-oxo-TEMPO, leading to an elimination of the spin of the nitroxide, which is called an ESR spin destruction method. The present ESR spin destruction experiments revealed that hematoporphyrin radical did not react with dGMP. On the other hand, photoexcited riboflavin reacted with dGMP to produce riboflavin anion radical and guanine cation radical. Guanine cation radical has been postulated to be produced by direct effect of radiation in DNA (29). The specific reactivity of photoexcited riboflavin with dGMP can be explained by the fact that guanine is most easily oxidized of the nucleic acid bases (30). The present results suggested that hematoporphyrin reacts specifically with guanine residues of single-stranded DNA via a Type II (1'O₂-involved) mechanism.
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whereas riboflavin reacts specifically with guanine residues of double-stranded DNA via a Type I (radical-involving) mechanism.

Kuratomi and Kobayashi (31) have presented evidence for binding of lumiflavin to DNA through the interaction of the flavin moiety with guanine bases of double-stranded DNA. The interaction by intercalation and/or hydrogen binding may be suitable for the electron transfer from guanine base to excited riboflavin. The 8-OH-dG formation and oxidative degradation at the 5' site of 5'-GG-3' sequences may be explained as shown in Fig. 8. The radical ion pair resulting from forward electron transfer from single guanine to photoexcited riboflavin easily reverts back to the ground state by back electron transfer within the complex (Mechanism A). However, with consecutive guanine residues, another electron transfer to the riboflavin-interacting guanine from the adjacent guanine occurs, resulting in much lower rate of back electron transfer, and subsequently in high efficiency of damage of the 5' located guanine (Mechanism B). In this mechanism, the electron transfer from 5' guanine to 3' guanine is expected to occur more readily than that from 3' guanine to 5' guanine. Although we have no direct evidence that would support this electron-transfer mechanism, Sugiyama et al. (32) have proposed that an intramolecular electron transfer from purine at 5'-side to an adjacent uracil-5-yl radical may occur in a specially oriented complex formed in the duplex. Alternatively, there remains a possibility that the specific cleavage is a consequence of more selective binding of riboflavin to 5'-GG-3' sequences, compared with single guanine residue (Mechanism C). This may be supported by the report that the interaction of lumiflavin with poly(dG)-poly(dC) was the strongest among the polynucleotides investigated, e.g. poly(dG), poly(dC), poly[d(A-T)], poly[d(A-T)], or DNA (31). The formed guanine cation radical could react with oxygen molecule and water molecule to form various kinds of oxidized products and 8-OH-dG, respectively. Relevantly, Cadet et al. (3) have suggested from experiments with ionizing radiation that guanine cation radical reacts with water to produce 8-OH-dG.

UV radiation-induced skin cancer is thought to arise from mutations in protooncogenes induced by the UV wavelengths present in solar light. It is considered that the mutation spectrum of UV light is consistent with the mutagenesis models of the premutagenic cyclobutane and pyrimidine (6-4) photoproducts induced by UV light (2,7,33-35). However, irradiation of cells by long UV wavelengths resulted in higher levels of mutation and transformation than would be expected on the basis of the pyrimidine photoproducts alone (36,37). Recently, Shibutani et al. (15) reported that the formation of 8-OH-dG causes misreplication of DNA that might lead to mutation or cancer. Furthermore, Cheng et al. (38) have illustrated mutagenic replication of 8-OH-dG as a template causing G→T substitution. The mutations at non-dipyrimidines (39) may be explained by 8-OH-dG formation and subsequent mutagenic replication. In addition, the similar mechanism might be responsible for nucleotide changes such as GGT→TGT and GGC→TGC in ras oncogenes, although these mutations have been generally explained by the mutagenesis models of pyrimidine photoproducts (2). The present data raise the possibility that irradiation with UV light longer than 300 nm of DNA in the presence of endogenous sensitizer causes 8-OH-dG formation as the critical event in the UV carcinogenesis.

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