reacts with DNA like a monofunctional analogue. However, the free ligand would be available to react with other molecules within the cell. This explains the relatively high levels of DNA–protein cross-links produced by this drug (Zwelling et al., 1979b). It is possible that some of these cross-links might labilize the drug from DNA. This may explain the report that *trans*-DDP-induced DNA–protein cross-links are removed in an excision repair deficient cell line (Gantt et al., 1984). Rather than postulate a new repair pathway, it is possible that the protein cross-links could have spontaneously dissociated from DNA. It is probable, however, that the major reaction of the monofunctional adducts in cells is with glutathione. That this is a significant reaction is supported by the observation that reducing glutathione levels in cells markedly enhances toxicity to *trans*-DDP but has little if any effect on *cis*-DDP toxicity (Andrews et al., 1985).

It has recently been proposed that the limited toxicity of *trans*-DDP is attributable to preferential repair of the DNA adducts as compared to repair of *cis*-DDP adducts (Ciccarelli et al., 1985). The majority of adducts were repaired in less than 6 h. The present report would suggest that the adduct rapidly repaired is a glutathione–Pt–DNA cross-link. However, such a repair process may contribute little to detoxifying the drug if the adducts are already detoxified by reaction with glutathione. *trans*-DDP-induced interstrand cross-links are also apparently repaired more rapidly than their *cis*-DDP counterparts (Plooy et al., 1984); however, the difference in repair rates is much less dramatic than the repair of total adducts in that the repair of interstrand cross-links took 24 h.

In summary, the ineffectiveness of *trans*-DDP is related to the high proportion of monofunctional adducts in DNA that rearrange very slowly to toxic bifunctional adducts. Persistent monofunctional adducts react rapidly with glutathione and become potentially less toxic. In addition, there is the possibility that some reactions may result in trans labilization of the drug from DNA, thereby rendering it innocuous.

Registry No. *trans*-DDP, 14913-33-8; glutathione, 70-18-8.

References

Andrews, P. A., Murphy, M. P., & Howell, S. B. (1985) *Cancer Res. 45*, 6250–6253.

Bradley, M. O., Patterson, S., & Zwelling, L. A. (1982) *Mutat. Res. 96*, 67–74.

Butour, J.-L., & Johnson, N. P. (1986) *Biochemistry 25*, 4534–4539.

Ciccarelli, R. B., Solomon, M. J., Varshavsky, A., & Lippard, S. J. (1985) *Biochemistry 24*, 7533–7540.

Eastman, A. (1982) *Biochemistry 21*, 6732–6736.

Eastman, A. (1983) *Biochemistry 22*, 3927–3933.

Eastman, A. (1986) *Biochemistry 25*, 3912–3915.

Eastman, A. (1987a) *Pharmacol. Therapeut.* (in press).

Eastman, A. (1987b) *Chem.-Biol. Interact.* (in press).

Gantt, R., Taylor, W. G., Camalier, R. F., & Stephens, E. V. (1984) *Cancer Res. 44*, 1809–1812.

Plooy, A. C. M., Fichtinger-Schepman, A. M. J., Shutte, H. H., van Dijk, M., & Lohman, P. H. M. (1985) *Carcinogenesis (London) 6*, 561–566.

Roberts, J. J., & Thomson, A. J. (1979) *Prog. Nucleic Acid Res. Mol. Biol. 22*, 71–133.

Zwelling, L. A., Filipski, J., & Kohn, K. W. (1979a) *Cancer Res. 39*, 4989–4995.

Zwelling, L. A., Anderson, T., & Kohn, K. W. (1979b) *Cancer Res. 39*, 356–369.

Sequence Context Effects on 8-Methoxypsoralen Photobinding to Defined DNA Fragments†

Evelyne Sage* and Ethel Moustacchi

Institut Curie, Biologie, 26 Rue d'Ulm, 75231 Paris Cedex 05, France

Received December 5, 1986; Revised Manuscript Received February 10, 1987

ABSTRACT: The photoreaction of 8-methoxypsoralen (8-MOP) with DNA fragments of defined sequence was studied. We took advantage of the blockage by bulky adducts of the 3′–5′-exonuclease activity associated with the T4 DNA polymerase. The action of the exonuclease is stopped by biadducts as well as by monoadducts. The termination products were analyzed on sequencing gels. A strong sequence specificity was observed in the DNA photobinding of 8-MOP. The exonuclease terminates its digestion near thymine residues, mainly at potentially cross-linkable sites. There is an increasing reactivity of thymine residues in the order T < TT ≪ TTT in a GC environment. For thymine residues in cross-linkable sites, the reactivity follows the order AT ≪ TA ~ TAT ≪ ATA < ATAT < ATATAA. Repeated A-T sequences are hot spots for the photochemical reaction of 8-MOP with DNA. Both monoadducts and interstrand cross-links are formed preferentially in 5′-TpA sites. Our results highlight the role of the sequence and consequently of the conformation around a potential site in the photobinding of 8-MOP to DNA.

Psoralens are a class of heterocyclic compounds (furocoumarins) which are used as photosensitizing agents in the treatment of various skin diseases (referred to as PUVA therapy). Psoralen plus UVA treatment has lethal and mutagenic effects on bacteria, yeast, and mammalian cells [for reviews, see Ben-Hur and Song (1984) and Averbeck (1985)]. It is also known to be carcinogenic in mice [for a review, see Ben-Hur and Song (1984)]. Nucleic acids are targets for the

† This work was financially supported by the CNRS, the CEA (Saclay, France), the ARC (Villejuif, France), and Contracts 852017 (INSERM) and B16-151-F (CCE, Bruxelles).

0006-2960/87/0426-3307$01.50/0

photoreaction of psoralens. This has made psoralens an excellent probe for chromatin and nucleic acid structure [reviewed in Cimino et al. (1985)]. The photobinding of psoralen to DNA is a multistep reaction [for reviews, see Dall'Acqua (1977), Song and Tapley (1979), Cimino et al. (1985), and Gasparro (1987)]. In the absence of light, psoralen intercalates in the double helix and forms a noncovalent complex with DNA. Upon irradiation with UVA light (320–400 nm), cycloaddition of psoralen to the 5,6 double bond of pyrimidine bases (mainly thymine) yields monoadducts. Three thymine monoadducts have been observed; two diastereomeric furan-side and a pyrone-side monoadducts. The proportion of each type varies with the different psoralen derivatives and the wavelength of irradiation (Straub et al., 1981; Kanne et al., 1982; Tessman et al., 1985). Absorption of a second photon by a furan-side monoadduct yields an interstrand DNA cross-link. Photoaddition provokes a distortion of the double helix which shows substantial unwinding and kinking at the site of the damage (Pearlman et al., 1985).

In vitro, *Escherichia coli* DNA polymerase I is able to copy across psoralen monoadducts but is blocked by cross-links (Piette & Hearst, 1983). In vivo, psoralen plus UVA treatment inhibits DNA synthesis and transcription [for a review see Ben-Hur and Song (1984)]. Bypass of monoadducts has been reported in yeast (Chanet et al., 1983). Photoadducts are repaired by the excision repair system; the removal of cross-links involves, in addition, a recombinational repair step [for a review, see Ben-Hur and Song (1984)]. For example, in *E. coli*, the *UVR ABC* and *Rec A* proteins are required (Seeberg, 1981; Sancar et al., 1985). In yeast, similar genes are responsible for the repair of psoralen- plus UVA-induced lesions (Moustacchi et al., 1983). These lesions are mutagenic. In *E. coli*, T → C, C → T transitions and also transversions are observed (Saffran & Cantor, 1984; Piette et al., 1985), as well as frame-shift mutations (Yoon, 1982; Piette et al., 1985). Comparison on repairability and mutagenic effects of monoadditions vs. biadditions in eucaryotic cells (Chanet el at., 1983; Moustacchi et al., 1983; Averbeck et al., 1984; Averbeck, 1985) was aimed at establishing a correlation between damage and photobiological activity. Parallel studies have been done with several psoralen derivatives in view of their potential use in photochemotherapy (Averbeck, 1985). The knowledge of the sites of the photoadducts can be expected to give some clues to better understand how mutations occur.

In this perspective, we have sought to visualize the damages induced by several psoralen derivatives on DNA fragments of defined sequence. We took advantage of the fact that the exonuclease associated with T4 DNA polymerase is blocked by bulky DNA adducts (Fuchs, 1984; Doetsch et al., 1985). The digestion products have been analyzed on sequencing gels. We have first demonstrated that the 3′–5′-exonuclease associated with DNA polymerase of phage T4 was a suitable tool to determine and quantitate the sites of psoralen monoaddition and interstrand cross-links in DNA. In this study, the bifunctional derivative 8-methoxypsoralen (8-MOP)[1] has been chosen as a model compound. A detailed analysis of the termination products has revealed that monoaddition, and biaddition as well, preferentially occurs at 5′-TpA cross-linkable sites. It has also revealed a strong sequence effect on the formation of photoadducts. Repeated AT sequences are hot spots for psoralen photobinding. Our results give more precise information on the sites of photoreaction than previous studies based on HPLC isolation of adducts and spectroscopic techniques.

Experimental Procedures

Chemicals and Enzymes. 8-[$^3H_3$]Methoxypsoralen was purchased from Amersham (specific activity 74 Ci/mmol). 8-Methoxypsoralen (8-MOP) was a commercial product from Sigma. The stock solution was at a concentration of 5×10^{-3} M in Me_2SO. [α-^{32}P]dNTP (specific activity 800 Ci/mmol) and [γ-^{32}P]ATP (specific activity 3000 Ci/mmol) were obtained from either Amersham or New England Nuclear. Restriction enzymes, T4 polynucleotide kinase, and T4 DNA polymerase were purchased from Bethesda Research Laboratories. Calf intestine alkaline phosphatase came from P-L Biochemicals and proteinase K from Boehringer, Mannheim.

DNA Preparation. Plasmid pMCI was prepared from transformed *E. coli* S90C (gift of Dr. D. E. Brash, Boston, MA) according to standard procedures. Phage M13 mp8 *lacI* 935 resulted from cloning of the first 935 bp of the *lacI* gene of *E. coli* (*Hin*cII digestion of the plasmid pMCI) at the *Hin*dIII site of the polylinker of M13 mp8 phage. The replicative form (RF) DNA of phages M13 mp8 and M13 mp8 *lacI* 935 was isolated by standard procedures from infected *E. coli* JM 109 or JM 101.

Preparation of 5′-^{32}P End-Labeled DNA Fragments. Ten micrograms of the purified M13 mp8 *lacI* 935 DNA was digested with *Hin*dIII restriction enzyme. The 5′ ends were labeled with [γ-^{32}P]ATP (250 μCi) using T4 polynucleotide kinase as described in Maxam and Gilbert (1980). A second digestion with *Nci*I gave rise to four end-labeled fragments, HN1, HN2, HN3, and HN4, of 25, 49, 76, and 540 bp, respectively. These fragments were purified as described (Doetsch et al., 1985).

Photobinding of Psoralen Derivatives to DNA. Aliquots of 5′ end-labeled DNA (approximately 6×10^4 cpm) were dissolved in 18–19 μL of 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA (TE buffer); 1 or 2 μL of 8-MOP in Me_2SO was added to give a final psoralen concentration of $(2.5–5) \times 10^{-4}$ M. Carrier DNA was avoided. The solution (20-μL droplets, on ice) was irradiated with a HPW 125 Philips lamp emitting mainly at 365 nm (UVA), at a fluence of 20 J·m^{-2}·s^{-1} as determined by a Black-Ray ultraviolet meter (Model J 221; Ultraviolet Products Inc., San Gabriel, CA). A Pyrex glass water filter assured that the contribution of wavelengths below 340 nm was negligible. It also protected the samples from temperature rises that could lead to evaporation. The radiation doses are given in the text. The exposures at 390 and 405 nm were performed by using a 2.5-kW Hg lamp in a Schoeffel housing with a Kratos 252 high-intensity grating monochromator (Averbeck & Cundari, 1986). Schott glass filters were used to eliminate transmission of UV light below 375 or 385 nm. The dose rate was 20 and 30 J·m^{-2}·s^{-1}, respectively.

The reacted DNA was freed from noncovalently bound 8-MOP molecules by four to six chloroform–isoamyl alcohol (19:1 v/v) extractions followed by ethanol precipitation. For each experiment, two control samples were done. One control was a DNA sample incubated with 8-MOP in the dark. For the second mock reaction, DNA was irradiated in 5–10% Me_2SO, in the absence of 8-MOP, at a dose corresponding to the maximal exposure of treated samples.

Digestion by T4 DNA Polymerase 3′–5′-Exonuclease of 8-MOP-Modified DNA. Treated samples were resuspended

[1] Abbreviations: bp, base pair(s); TCA, trichloroacetic acid; Me_2SO, dimethyl sulfoxide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; dNTP, deoxyribonucleotide triphosphates; ds, double stranded; ss, single stranded; cpm, counts per minute; PR, photoreversion; XL, cross-link; MA, monoadduct; 8-MOP, 8-methoxypsoralen; HPLC, high-performance liquid chromatography; Py, pyrimidine; Pu, purine.

in a minimal volume of doubly distilled water. One fourth of each sample was used as control without further digestion. The remaining part of each sample was digested in 20 μL of 33 mM Tris–acetate, pH 7.8, 10 mM magnesium acetate, 66 mM potassium acetate, 0.5 mM dithiothreitol, and 0.1 mg/mL bovine serum albumin with 3.5 units of T4 DNA polymerase for 2 h at 37 °C. In order to avoid a DNA polymerization reaction, all experiments were performed in the absence of dNTP. The reaction was terminated by adding 1 μg of carrier DNA and 0.3 M sodium acetate followed by two extractions with chloroform–isoamyl alcohol (19:1) and ethanol precipitation.

Photoreversion. Before electrophoresis of the DNA fragments modified by 8-MOP and digested with T4 DNA polymerase exonuclease, most of the interstrand cross-links were photoreversed according to Straub et al. (1981) by a UV dose of 6 kJ·m^{-2} at 254 nm. The irradiation was done on DNA containing droplets of 5 μL in TE buffer, which afterward were evaporated.

Analysis of Damaged Sites by Sequencing Gel Electrophoresis. The samples were worked up for high-resolution sequence gel analysis as described by Maxam and Gilbert (1980). The polyacrylamide concentrations of the gels varied between 12% and 20% according to the length of the DNA fragments to be analyzed. The samples were loaded on gels together with controls and with the four Maxam and Gilbert sequencing reaction products of unmodified DNA. The T4 DNA polymerase associated exonuclease termination products are visualized as bands on autoradiograms. They are quantified by measuring by Cerenkov counting the radioactivity in each gel band relative to the total radioactivity in the lane. To account for more than one photoproduct per DNA strand, a correction factor was applied as described by Gordon and Haseltine (1980).

Results

Suitability of T4 DNA Polymerase 3′–5′-Exonuclease for Mapping and Quantitation of Psoralen DNA Adducts. The 3′–5′-exonuclease activity associated with the phage T4 DNA polymerase is blocked by mono- and biadducts photoinduced by 8-MOP. Indeed, less than 5% of radioactivity is released from a linear plasmid pMCI treated with 8-[^{3}H]MOP plus UVA and digested for 2 h by the enzyme. The kinetics of hydrolysis of a 5′-^{32}P end-labeled fragment treated with 8-MOP plus UVA demonstrate a plateau starting at 30 min. In subsequent experiments, a digestion period of 2 h was adopted on the basis of such kinetics. The dose–response of the exonuclease activity was determined on the 5′ end-labeled HN3 DNA fragment exposed to 8-MOP and different doses of UVA. At the end of the enzymatic digestion, aliquots were assayed for TCA precipitation. Figure 1 shows a linear increase of the undigested fraction as a function of dose. From these results, it is clear that the exonuclease activity of T4 DNA polymerase is suitable for mapping and quantitating psoralen DNA adducts.

Use of Sequencing Gels in Combination with Enzymatic Digestion for Visualization and Quantitation of Cross-Links and Monoadducts. 5′ end-labeled double-strand DNA of defined sequences (fragments HN2 or NH3 of 49 or 76 bp, respectively) was exposed to 8-MOP and several doses of UVA. DNA samples were then digested with T4 DNA polymerase 3′–5′-exonuclease. Digestion products were resolved by polyacrylamide gel electrophoresis under denaturing conditions. The Maxam and Gilbert sequencing reactions were performed on the corresponding intact DNA. Samples were coelectrophoresed, in addition to undigested 8-MOP-treated DNA

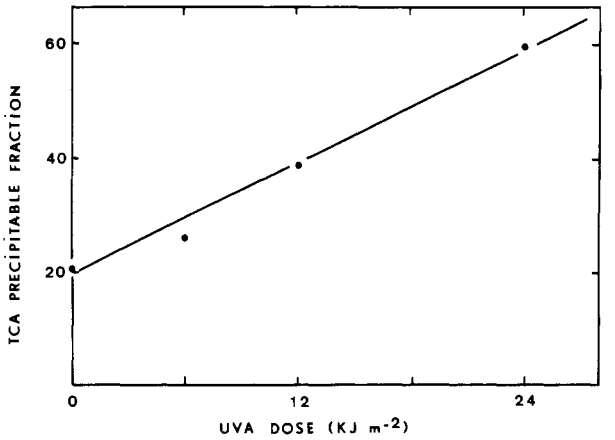

FIGURE 1: Hydrolysis of 8-MOP-modified DNA by T4 DNA polymerase 3′–5′-exonuclease as a function of UVA dose. A 5′ end-labeled DNA fragment, HN3 (76 bp), was exposed to 8-MOP and to different doses of 365-nm irradiation. After 2 h of hydrolysis with the exonuclease, aliquots were TCA precipitated on Whatman 3MM filters. Filters were washed, dried, and counted in an LKB 1216 Rackbeta II spectrometer after addition of scintillation fluid. The ^{32}P-TCA-precipitable fraction is given as a function of the dose.

samples. Two autoradiograms of gels are shown in Figure 2. Much qualitative and quantitative information can be drawn from such experiments. Lanes 13–14 (Figure 2a), referring to unmodified and digested DNA samples, show no band pattern. Some radioactivity migrates as a single-stranded DNA fragment (undigested molecules). In lanes 10–12 (Figure 2a), referring to modified and digested DNA samples, a large amount of radioactivity migrates as double-stranded molecules. These are due to cross-linked fragments, either undigested or partially digested. The sequence band pattern, which is of weak intensity in these lanes, represents termination events at monoadducts. If after enzymatic digestion and before loading the samples on the gel the cross-links are photoreversed by irradiation at 254 nm, most of the cross-links are eliminated, and new bands appear, mainly on the 3′ side (see Figure 2a, lanes 10–12 as opposed to lanes 5–7). These bands may reflect termination sites at monoadducts as well as biadducts. This result indicates that 8-MOP photoadducts should be photoreversed after digestion with the exonuclease, in order to be able to see all the termination events. We verified on undigested, 8-MOP-treated or untreated DNA samples that UV irradiation at 254 nm did not produce by itself any cleavage of the DNA fragments.

When the untreated and 8-MOP-treated DNA samples are loaded on gel, without digestion by the exonuclease and without photoreversion of cross-links, no sequence band pattern is observed (data not shown). Furthermore, the proportion of cross-links can be estimated. The amount of radioactivity migrating as single-stranded DNA fragments decreases with increasing UVA doses, whereas the amount of radioactivity migrating as double-stranded DNA fragments increases. This result confirms that more and more cross-links are formed upon irradiation. The formation of monoadducts and cross-links on identical DNA molecules follows a Poisson distribution. The ratio R, $[\text{cpm ds/cpm (ds + ss)}]_{\text{treated sample}} - [\text{cpm ds/cpm (ds + ss)}]_{\text{control sample}}$, is the measure of the proportion of DNA molecules with at least one cross-link. $1 - R$ represents the proportion of un-cross-linked molecules. According to a Poisson distribution, an average value of one cross-link per molecule means that 37% of the molecules are un-cross-linked. $1 - R$ is the second term in the Poisson series and gives the mean value of the number of cross-links per DNA molecule. The estimation of the proportion of cross-links was

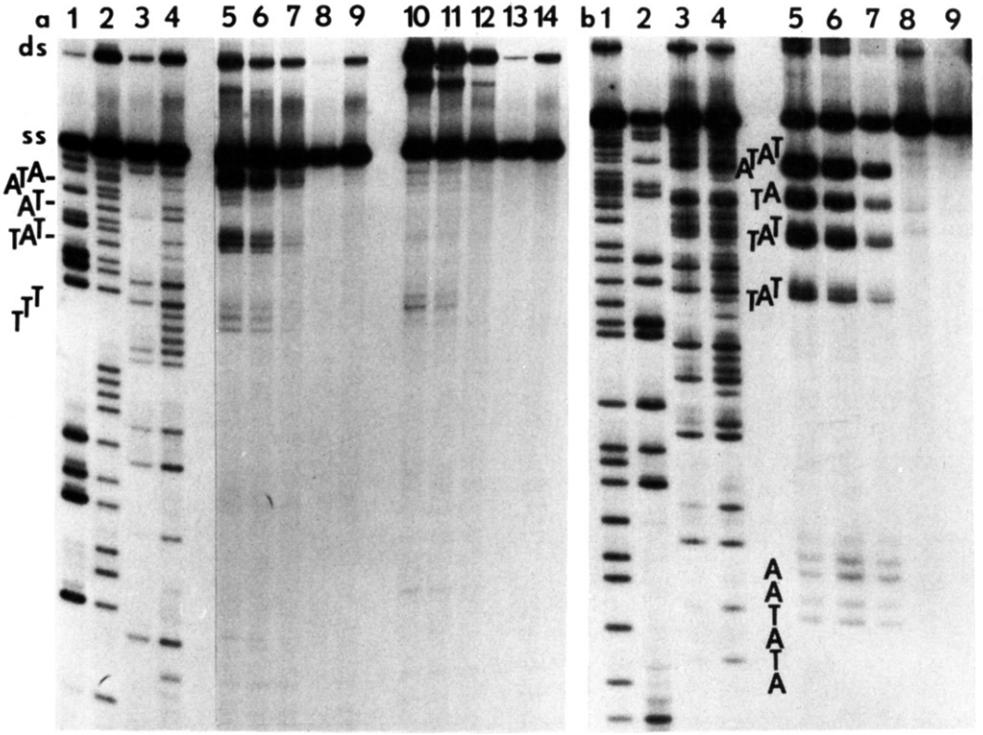

FIGURE 2: Analysis of the sites of photoadditions of 8-MOP on HN2 DNA fragment (a) and HN3 DNA fragment (b). 5′ end-labeled DNA was irradiated at 365 nm at a dose of 24 kJ·m^{-2} (lanes 9 and 14) or incubated with 2.5×10^{-4} M 8-MOP and irradiated at 365 nm at doses of 0 (lanes 8 and 13), 6 (lanes 7 and 12), 12 (lanes 6 and 11), and 24 kJ·m^{-2} (lanes 5 and 10). After a 2-h digestion with the T4 DNA polymerase 3′–5′-exonuclease, termination products are resolved on 20% sequencing gels. In lanes 5–9, photoreversion of adducts (irradiation at 254 nm at a dose of 6 kJ·m^{-2}) was performed before the samples were loaded on the gel. The Maxam and Gilbert sequencing reaction products for G, G + A, C, and C + T are represented in lanes 1, 2, 3, and 4 in (a) and in lanes 2, 1, 3, and 4 (b), respectively.

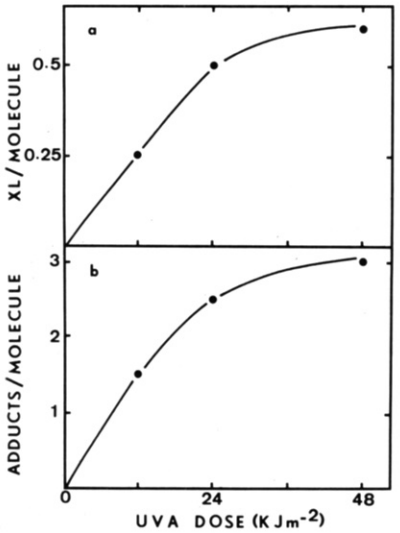

FIGURE 3: Amount of cross-links (a) and total photoadducts (b) produced in DNA fragments as a function of UVA doses. These calibration curves are derived from gels obtained according to conditions described in the text.

systematically done for each experiment. A typical quantitation is shown in Figure 3a.

The fully digested molecules of untreated or 8-MOP-modified DNA samples migrate as short oligonucleotides (di- or trideoxynucleotides). They represent unmodified molecules. As for the quantification of cross-links, the ratio of cpm in low bands to total cpm in the lane gives access to the number of DNA molecules carrying at least one photoadduct. Figure 3b shows the values obtained for a typical experiment. The formation of cross-links parallels the formation of total adducts upon irradiation. This can be explained as follows. For the photobinding of 8-MOP to DNA, 8-MOP is at the limit of its solubility in the reaction mixture. There is a large excess of psoralen compared to DNA. The concentration of free drug varies very little during the photobinding. Under these conditions, prior to photoreaction, there is a maximal intercalation of the drug depending only on the dissociation constant K_D: $K_D = [P][S]/[PS]$ where P is 8-MOP, S is the intercalation site, and PS is the intercalated drug. The amount of occupied sites $[PS]/[S]$ becomes independent of the DNA concentration [given as 0.14 for 8-MOP at a concentration of 1.8×10^{-4} M in Hearst (1981)].

The photobinding will depend only on the ratio ϕ_2/ϕ_3, where ϕ_2 is the quantum yield for photoaddition and ϕ_3 the quantum yield for photobreakdown of psoralen. For 8-MOP, this ratio is 24 (Hearst, 1981) so that the photobinding is largely favored. This explains that during long exposure to 365-nm light monoadditions continue to be induced, while some monoadducts absorb a second photon to form cross-links.

The reaction conditions [$(2.5–5) \times 10^4$ M psoralen, close to saturation for most derivatives] were set in view of experiments with other psoralen derivatives which have very different values for solubility, K_D, ϕ_2, and ϕ_3. The DNA target is small, and a maximum of intercalation is needed. Then the radiation dose is adjusted to obtain about one or two photoadducts per DNA fragment.

A sequence band pattern for monoadducts (Figure 2a, lanes 10–12) and monoadducts plus biadducts (Figure 2a,b, lanes 5–7) is observed. Comparison with the sequencing ladder allows the assignment of enzymatic termination to modified sites. Qualitatively, it is seen that bands are aligned with bases close to potential psoralen binding sites, i.e., thymine residues mainly in cross-linkable sites. Several bands correspond to a given site. Moreover, the bands corresponding to termination events are shifted, compared to sequencing T reaction bands. Indeed, even a simple site like AT or TA exhibits at least two

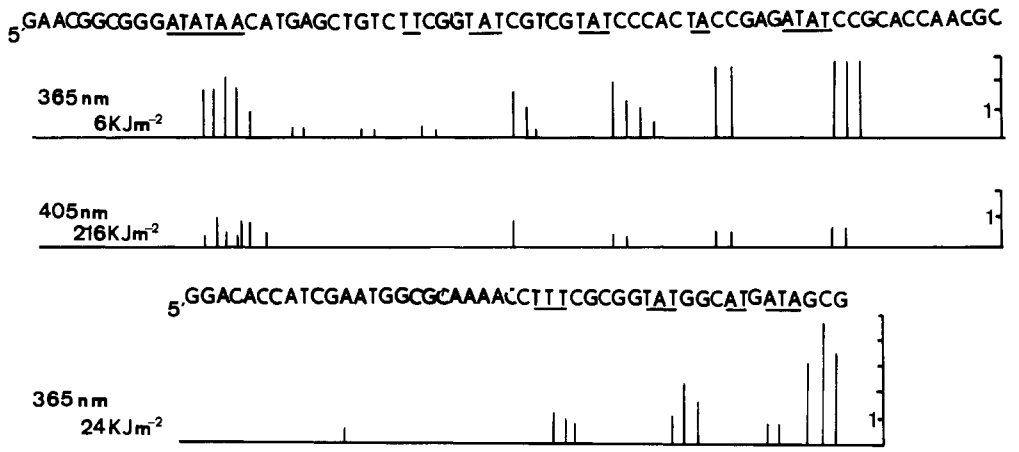

FIGURE 4: Distribution of 8-MOP adducts along HN2 and HN3 DNA fragments. The height of bars reflects the intensity of corresponding bands and represents the percentage of termination products calculated as described under Experimental Procedures. The height of bars can be compared inside a line, but not between lines, because the extent of modification per DNA molecule is not the same in the three experiments. The position of bars corresponds to the location of bands on autoradiograms. When irradiation of DNA–8-MOP is done at 365 nm, the diagram represents the band pattern obtained after photoreversion of adducts.

termination events. The possible origins of this complex band pattern are discussed later. The shift of bands varies between 1.5 and 2.5 nucleotides, from one site to an other. It takes into account the base which is eliminated by the sequencing process, and the absence of the 3′-phosphate terminus after digestion with T4 DNA polymerase 3′–5′-exonuclease. In addition, the mobility of a termination product may be slowed down by the presence of the psoralen addition and/or by the presence of an extra nucleotide on the 3′ side of a modified thymine residue. In Figure 2a, it appears clearly that, when adducts are photoreversed, the termination products migrate more rapidly. In addition, new bands appear at certain cross-linkable sites. Some of them are probably representative of stops of the exonuclease at biadducts encountered by the enzyme before any monoadduct.

Mapping, Quantification, and Sequence Specificity of the Photoproducts. Slices corresponding to isolated bands or group of bands (as seen in Figure 2a,b) were cut out of the gels and counted for radioactivity. The percentage of termination products at each site was determined as indicated under Experimental Procedures. The results are shown in Figure 4. The following conclusions can be drawn. First, all thymine residues are not reactive. Single thymines in a GC environment are very poorly reactive. Adjacent thymines are preferred, TTT $\gg$ TT > T when surrounded by GC. Second, most of the termination events correspond to thymine residues located in potential cross-linkable sites, AT or TA. A multiple site is strongly preferred. The order of reactivity of the studied sites is 5′-TAT < ATA < ATAT < ATATAA. Multiple cross-linkable sites are hot spots for 8-MOP photobinding. Third, it appears on both DNA fragments that 5′-ApT is a very weak site. Photoadditions are strongly preferred in 5′-TpA cross-linkable sites. A site like 5′-TAT, which has two thymine residues, is about equivalent to 5′-TpA and is at least 2 times weaker than the site 5′-ATA. This observation is additional proof that photoadducts are preferentially formed in 5′-TpA.

When a wavelength higher than 395 nm is used for irradiation, diadducts are formed only at 5–10% (Tessman et al., 1985). The dark complex 8-MOP–DNA was irradiated at 390 or 405 nm as described under Experimental Procedures. Photoadducts were mapped as above, except that there was no irradiation at 254 nm for photoreversion. Because there was no photoreversion of adducts after the enzymatic digestion, all the bands reflect termination events at monoadducts.

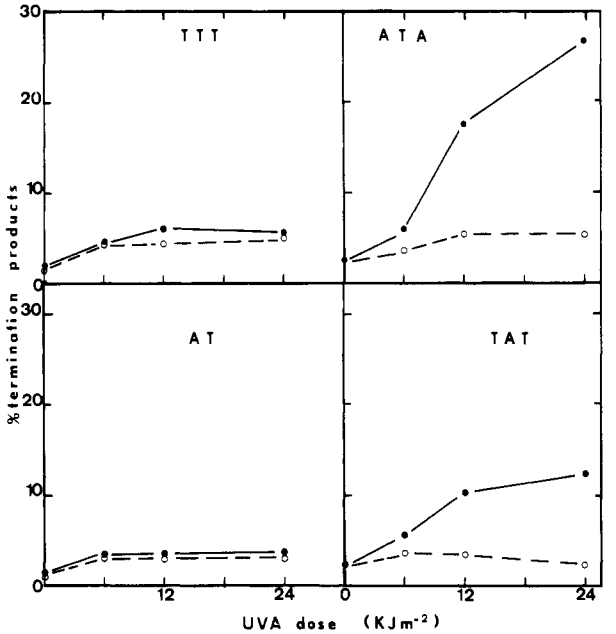

FIGURE 5: Dose–response of termination products appearing on autoradiograms with (●) or without (○) photoreversion of cross-links. It represents the quantification done on the gel presented in Figure 2a.

Figure 4 shows that the band pattern obtained when mainly monoadducts are formed parallels the band pattern obtained after irradiating the samples at 365 nm. In addition to the preceding results, it is a formal proof that monoadducts are formed mainly at cross-linkable sites.

Sites of Cross-Link Formation. The cross-link formation can be evidenced in analyzing the digestion products on the gel, with and without photoreversion of the cross-links as in Figure 2a. Quantification is given in Figure 5, which represents the formation of photoadducts at four sites, related to the UVA dose. In this experiment, the amount of molecules with at least one cross-link is estimated to 6%, 16%, and 34% for UVA doses of 6, 12, and 24 kJ·m^{-2}, respectively. It fits with about two adducts per molecule for a UVA dose of 24 kJ·m^{-2}. When samples are not photoreversed for cross-links, the detection of monoadducts is masked by the increasing amount of cross-linked molecules induced as a function of the UVA dose. From molecules carrying the two types of adducts, the photoreversion may liberate termination products at bi-

adducts as well as at monoadducts.

Let us examine the situation at each of the four reactive sites (i.e., TTT, TAT, AT, and ATA). At the TTT site, photoreversion does not cause more termination products to appear. Indeed, no biadducts are possible at this location. It also indicates that no cross-linked sites are formed on the 5′ side of this site (at the two AT sites, for example). The increased amount of termination events appearing at the TAT site after photoreversion comes from cross-links formed at this site. Note also the presence of a new band at this site (Figure 2a, lanes 10–11 as opposed to lanes 5–6). The intensity and number of bands corresponding to the AT site are unchanged when photoreversion was done. It confirms the absence of cross-link formation at this site. Inversely, there is a great increase in termination events appearing at the ATA site upon photoreversion. New bands are visible for high UVA doses (Figure 2a, lanes 10–11 and 5–6). Reversion of adducts formed after exposure to 12 and 24 kJ·m^{-2} UVA light may liberate termination products at monoaddition on the ATA site from molecules which are cross-linked at the TAT site. It may as well liberate termination products at biadducts formed at the ATA site. TAT and ATA sites are obviously favored sites for the photoreaction. The greatest reactivity of the ATA site is probably due to a different environment of the thymine residues in the two sites. Taken together, these results show that 8-MOP biadditions do not occur at 5′-ApT sites. It indicates again that monoadducts as well as biadducts are preferentially formed in 5′-TpA sites, whereas 5′-ApT sites are poorly reactive.

Discussion

Mapping of damages induced in DNA of defined sequence by chemical or physical agents has been used to identify DNA lesions and to study the sequence specificity in the formation of these lesions in DNA (D'Andrea & Haseltine, 1978; Lippke et al., 1981; Henner et al., 1983; Muench et al., 1983; Sage & Haseltine, 1984; Doetsch et al., 1985; Duplaa & Téoule, 1985; Sawadaishi et al., 1986). Mapping of DNA adducts has also served as a tool to study the correlation between the distribution of DNA lesions and mutations (Brash & Haseltine, 1982; Bourre & Sarasin, 1983; Fuchs, 1984; Bichara & Fuchs, 1985; Glickman et al., 1986) as well as to study the specificity of DNA repair enzymes (Haseltine et al., 1980; Sancar et al., 1985; Helland et al., 1986).

The 3′–5′-exonuclease activity of the DNA polymerase of phage T4 has been used to determine qualitatively and quantitatively the distribution of cyclobutane pyrimidine dimers and (6–4) photoproducts (Doetsch et al., 1985), (acetylamino)fluorene adducts (Fuchs, 1984), and benzo[*a*]pyrenediol epoxide DNA adducts (E. Sage, unpublished results). The 3′–5′-exonuclease of the T4 DNA polymerase is blocked by bulky adducts. We have used this assay to map the 8-MOP adducts on two DNA fragments of known sequence and to study the sequence specificity in the photoreaction of 8-MOP with DNA.

The experiments presented here demonstrate that T4 DNA polymerase associated exonuclease can be used to locate and quantitate psoralen adducts on short DNA fragments. The average number of modified bases has been one or two per strand of DNA molecule for most of the experiments. The proportion of cross-links has been estimated to be around 20% of the total modifications upon irradiation at 365 nm. We observe that the order of reactivity of thymines in a GC environment is T < TT ≪ TTT and the order of reactivity of cross-linkable sites is AT ≪ TA ~ TAT ≪ ATA < ATATCC < ATATAA.

Depending on the photoreversion of cross-links, our assay reveals the formation of monoadducts or monoadducts plus biadducts. Using different wavelengths to irradiate the dark complex DNA–8-MOP, we have the possibility to study mainly monoadducts (Tessman et al., 1985). In our assay, it can be ruled out that stops of the T4 DNA polymerase associated exonuclease result from adducts on the unlabeled strand. In that case, a series of four bands corresponding to the four adjacent thymine residues on the unlabeled strand in the 49 bp DNA fragment would be observed.

Preferential Occurrence of Monoaddition at 5′-TpA Cross-Linkable Sites. The diagram in Figure 4 allows a direct comparison of the reactivity of different sites in the DNA fragments. Comparing the photobinding at AT and TA sites on both DNA fragments, it clearly appears that monoadditions occur preferentially in cross-linkable 5′-TpA sites, confirming the results obtained by Tessman et al. (1985). When the reactivity of another bifunctional psoralen derivative, HMT [4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen], toward two restriction sites, *Bam*HI (GGATCC) and *Kpn*I (GGTACC) in SV-40 DNA and synthetic linkers was analyzed, the same specificity was obtained (Gamper et al., 1984). It seems a general rule for psoralen derivatives (our unpublished experiments). One of the possible explanations for this preference is that 8-MOP intercalates better in 5′-TpA. According to Sobell et al. (1982), intercalative drugs, like acridine orange and ethidium ion, intercalate preferentially in 5′-pyrimidine-purine sequences in dinucleotides. The base stacking is different in sequences 5′-Py-Pu and 5′-Pu-Py and so is the stability of the structure.

Sequence Context Effect on Photoaddition. Our study gives a direct proof of the favored photoreaction at 5′-TpA site. It also shows the strong heterogeneity in the distribution of 8-MOP adducts along a DNA sequence. The 5′-TpA sites are not equivalent. Several examples demonstrate the importance of the flanking sequence on adduct formation at a cross-linkable site. Two identical 5′-TAT sites on the 76 bp DNA fragment exhibit a difference in reactivity. Comparison of adduct formation at the two sites, 5′-TAT and 5′-ATA, in the labeled chain of the 49 bp DNA fragment shows a favored photoreaction of 8-MOP on the thymine surrounded by two adenine residues (5′-ATA). This is confirmed by an experiment where the same DNA fragment is 3′ end labeled on the complementary strand and exposed to 8-MOP plus UVA, and where digestion by the 3′–5′-exonuclease of λ phage reveals a strong stop site at 3′-ATA which is the complementary sequence of 5′-TAT (data not shown). In this last assay, no real stops are observed at positions 3′-TA and 3′-TAT of the labeled strand.

The potential monoadducts at the TAT/ATA site are shown in Figure 6; the two strands have in principle the same potentiality in forming photoadditions with 8-MOP. The results show that this is not the case. Two hypotheses can be considered to explain this difference. (i) A preferential orientation of psoralen rings in the dark complex may be involved. For instance, the furan side could be directed preferentially toward a thymine bordered by two adenine residues. (ii) The bases bordering a reactive site may influence the photoreaction. An adenine residue on the 5′ side of site 5′-TpA confers a higher reactivity to the thymine than any other base. This assumption seems reasonable, since in the DNA fragment HN3 (76 bp) the site 5′-ATATC is more reactive than the two sites 5′-GTATC (see Figure 4). It is also in good agreement with the poor reactivity of a thymine flanked by C and G. This implies that 8-MOP will preferentially photoreact in $(AT)_n$ sequences.

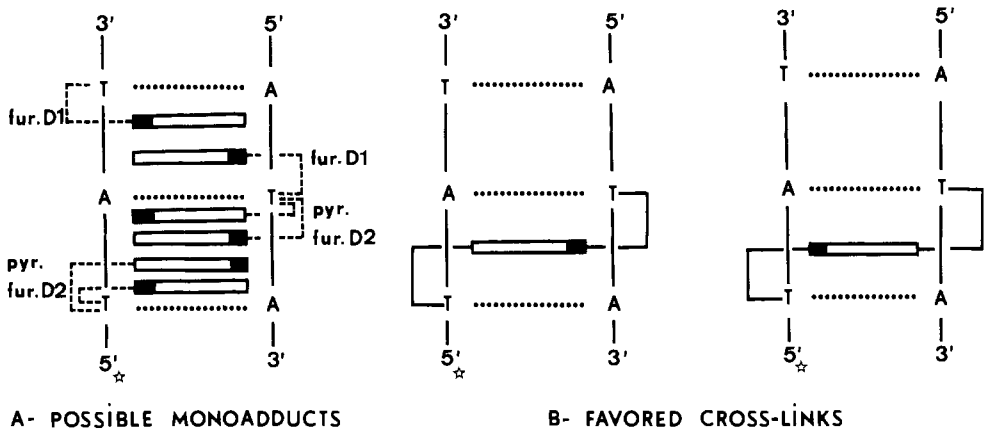

FIGURE 6: Schematic representation showing 8-MOP photoadduct formation at the 5′-TAT site. (a) Possible monoadducts at each thymine residue. (b) The two favored diadduct enantiomers. The adduct distribution in 8-MOP–DNA (calf thymus) irradiated at 365 nm is 28% of the furan-side thymine MA D2 (in 5′-TpX), 16% of the furan-side thymine MA D1 (in 5′-XpT), 19% of the pyrone-side MA, 2% of the furan-side cytosine MA, and 26% of the diadduct (Kanne et al., 1982).

Repeated AT Sequences Are Hot Spots for Photoreaction of 8-MOP. Beside the preferential photoreaction of 8-MOP in cross-linkable sites and more precisely in 5′-TpA, there is an increment in the reactivity in multiple cross-linkable sites. For example, as mentioned above, the site 5′-ATATC is more reactive than the site 5′-GTATC. A better example is given with the hot spot 5′-ATATAA in HN3 DNA which is more reactive than site 5′-ATATCC. The former sequence contains an extra cross-linkable site. It is probably a major cause for this difference in reactivity. The local stability of the double helix may also play a role. Calculation of the free energy ΔG_{total} according to Breslauer et al. (1986) for the dodecamers including the sites 5′-GGGATATAACAT-3′ and 5′-GAGATATCCGCA-3′ (ΔG_t of 24.2 and 27 kcal/mol, respectively) predicts a lower stability for the former site that may partly account for its greater reactivity toward 8-MOP plus UVA.

The structure of the double helix in $(AT)_n$ regions, known to have great flexibility (Widom, 1984), may assure a better overlap of the reactive atoms of intercalated 8-MOP (3,4- and 4′,5′-carbons) with the 5,6 double bond of thymine which favors photoreaction at these sites. Such sequences will be the favorite target for biadditions, since they allow the formation of a kink at the site of a cross-link (Pearlman et al., 1985). From our results and others, it is difficult to decide between a preferential intercalation or a preferential photoreaction in $(A\text{-}T)_n$ regions. The local conformation around thymine residues is important since the sequence specificity in the photoreaction of HMT on DNA is completely abolished when DNA is single stranded (Piette & Hearst, 1985). Our results highlight the role of sequence and conformation around a potential site in the photobinding of 8-MOP to DNA.

Occurrence of Biaddition at 5′-TpA Sites. Our results clearly show that biadditions occur at 5′-TpA sites. Indirect evidence from Zhen et al. (1986) is in accord with our observation. In contrast, we do not detect diadducts at 5′-ApT sites which are potential cross-linkable sites (Figure 5). The furan-side monoadduct D1 would mainly occur at this site; it is not a major adduct. In the experiments described in Tessman et al. (1985), this adduct is a poor contributor to the formation of cross-links. The dT–8-MOP–dT diadducts arise almost exclusively from the furan-side monoadduct D2, in agreement with our results. Figure 6 indicates that diadducts have a high probability to be formed in 5′-TpA sites.

Tentative Identification of Bands. The T4 DNA polymerase associated 3′–5′-exonuclease produces several termination products corresponding to a given thymine residue. One reasonable explanation is that it reflects the formation of the different photoadducts. Depending on the intercalation of the psoralen on one face or the other of the thymine ring, the exonuclease may stop at the modified base or one nucleotide before. An early stop can also be expected when the exonuclease meets a cross-link. However, the number of bands at a site and their intensities do not reflect exactly the probability of formation of the different photoadducts. Some of the bands can surely be attributed to the presence of certain monoadducts. It cannot be completely ruled out that cytosine residues adjacent to A-T-rich sites participate in the production of termination products. The configuration of adducts and the local conformation at a modified site may influence the enzymatic activity of the T4 DNA polymerase 3′–5′-exonuclease. It has been reported that a cross-link introduces a conformational change at 3′ of the cross-linked bases and a kink in the double helix (Peckler et al., 1982; Pearlman et al., 1985). A monoadduct is expected to produce a smaller perturbation, i.e., as at a pyrimidine dimer (Pearlman et al., 1985). No such complexity of band pattern has been reported for UV (254 nm) modified or AAF-modified DNA (Fuchs, 1984; Doetsch et al., 1985). Nevertheless, examining the band pattern obtained when adducts are or are not photoreversed, we see the bands of cross-links appearing on the 3′ side of certain sites (Figure 2a). An evolution of the intensity of bands at certain sites upon irradiation is also observed. This is in favor of an isomerization of the monoadducts. A photoisomerization of a furan-side monoadduct (mainly D2) to a pyrone-side monoadduct has been proposed (Tessman et al., 1985). Our assay should in principle be a powerful tool to detect the different types of photoadducts at any site.

Conclusion

We have addressed the question on the effect of the sequence context on the photocycloaddition of 8-MOP to thymine targets in DNA. We have brought a direct proof of preferential monoaddition and also biaddition at 5′-TpA cross-linkable sites. We have observed a strong sequence specificity of photoadduct formation on thymine residues flanked by adenine residues or located in A-T clusters. A thymine residue in a GC environment is a very poor target. By analyzing a number of DNA fragments of known sequence for photoaddition, it becomes possible to deduce "rules" that govern the reactivity of sites. The prediction of photobinding of 8-MOP and other psoralen derivatives in general is important in view of understanding their biological activities. The TATA boxes are likely to be preferential targets, and more generally any $(ApT)_n$ regions. Studies on the photobinding of 8-MOP and

its derivatives to certain sequences may help to better understand the mutagenic effect of these compounds.

ACKNOWLEDGMENTS

We thank Professor Claude Helene and Dr. Dietrich Averbeck for their critical reading of the manuscript. Special thanks are due to Claude Gardin for many enthusiastic discussions.

REFERENCES

Averbeck, D. (1985) *Mutat. Res. 151*, 217–233.
Averbeck, D., & Cundari, E. (1987) *Photochem. Photobiol. 45*, 371–379.
Averbeck, D., Papadopoulo, D., & Quinto, I. (1984) *Natl. Cancer Inst. Monogr. No. 66*, 127–136.
Ben-Hur, E., & Song, P. S. (1984) *Adv. Radiat. Biol. 11*, 131–171.
Bichara, M., & Fuchs, R. P. P. (1985) *J. Mol. Biol. 183*, 341–351.
Bourre, F., & Sarasin, A. (1983) *Nature (London) 305*, 68–70.
Brash, D. E., & Haseltine, W. A. (1982) *Nature (London) 298*, 189–192.
Breslauer, K., Frank, R., Blöcker, H., & Marky, L. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3746–3750.
Chanet, R., Cassier, C., Magaña-Schwencke, N., & Moustacchi, E. (1983) *Mutat. Res. 112*, 201–214.
Cimino, G., Gamper, H., Isaacs, S., & Hearst, J. (1985) *Annu. Rev. Biochem. 54*, 1151–1193.
Dall'Acqua, F. (1977) in *Research in Photobiology*, pp 245–255, Plenum Press, New York.
D'Andrea, A. D., & Haseltine, W. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4120–4124.
Doetsch, P., Chan, G., & Haseltine, W. A. (1985) *Nucleic Acids Res. 13*, 3285–3304.
Duplaa, A. M., & Têoule, R. (1985) *Int. J. Radiat. Biol. 48* 19–32.
Fuchs, R. P. P. (1984) *J. Mol. Biol. 177*, 173–180.
Gamper, H., Piette, J., & Hearst, J. (1984) *Photochem. Photobiol. 40*, 29–34.
Gasparro, F. P. (1987) in *Psoralen DNA Photobiology* (Gasparro, F. P., Ed.) CRC Press, Boca Raton, FL (in press).
Glickman, B. W., Schaaper, R. M., Haseltine, W. A., Dunn, R. L., & Brash, D. E. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6945–6949.
Gordon, L. K., & Haseltine, W. A. (1980) *J. Biol. Chem. 255*, 12047–12050.
Haseltine, W. A., Gordon, L. K., Lindan, C., Grafstrom, R., Shaper, N., & Grossman, L. (1980) *Nature (London) 285*, 634–641.
Hearst, J. (1981) *J. Invest. Dermatol. 77*, 39–44.
Helland, D., Doetsch, P., & Haseltine, W. A. (1986) *Mol. Cell. Biol. 6*, 1983–1990.
Henner, W. D., Rodriguez, L., Hecht, S., & Haseltine, W. A. (1983) *J. Biol. Chem. 258*, 711–713.
Kanne, D., Straub, K., Rapoport, H., & Hearst, J. (1982) *Biochemistry 21*, 861–871.
Lippke, J., Gordon, L. K., Brash, D. E., & Haseltine, W. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3388–3392.
Maxam, A., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499–560.
Moustacchi, E., Cassier, C., Chanet, R., Magaña-Schwencke, N., Saeki, T., & Henriques, J. A. P. (1983) in *Cellular Responses to DNA Damage* (Friedberg, E. C., & Bridges, B. A., Eds.) pp 87–106, Alan R. Liss, New York.
Muench, K., Misra, R., & Humayun, A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6–10.
Pearlman, D., Holbrook, S., Pirkle, D., & Kim, S. H. (1985) *Science (Washington, D.C.) 227*, 1304–1308.
Peckler, S., Graves, B., Kanne, D., Rapoport, H., Hearst, J., & Kim, S. H. (1982) *J. Mol. Biol. 162*, 157–172.
Piette, J., & Hearst, J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5540–5544.
Piette, J., & Hearst, J. (1985) *Int. J. Radiat. Biol. 48*, 381–388.
Piette, J., Decuyper-Debergh, D., & Gamper, H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7355–7359.
Saffran, W., & Cantor, C. (1984) *Nucleic Acids Res. 12*, 9237–9248.
Sage, E., & Haseltine, W. A. (1984) *J. Biol. Chem. 259*, 11098–11102.
Sancar, A., Franklin, K., & Sancar, G. (1985) *J. Mol. Biol. 184*, 725–734.
Sawadaishi, K., Miura, K., Ohtsuka, E., Ueda, T., Shinriki, N., & Ishizaki, K. (1986) *Nucleic Acids Res. 14*, 1159–1169.
Seeberg, E. (1981) *Mutat. Res. 82*, 11–22.
Sobell, H. M., Sakore, T. D., Jain, S. C., Banerjee, K. K., Bhandary, K. K., Reddy, B. S., & Lozansky, E. D. (1982) *Cold Spring Harbor Symp. Quant. Biol. 47*, 293–314.
Song, P. S., & Tapley, J. K. (1979) *Photochem. Photobiol. 29*, 1177–1197.
Straub, K., Kanne, D., Hearst, J., & Rapoport, H. (1981) *J. Am. Chem. Soc. 103*, 2347–2355.
Tessman, J., Isaacs, S., & Hearst, J. (1985) *Biochemistry 24*, 1669–1676.
Widom, J. (1984) *Nature (London) 309*, 312–313.
Yoon, K. (1982) *Mutat. Res. 93*, 253–262.
Zhen, W., Buchardt, O., Nielsen, H., & Nielsen, P. E. (1986) *Biochemistry 25*, 6598–6603.