# Photoreactivities and Thermal Properties of Psoralen Cross-Links†

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ABSTRACT: We have studied the photoreaction of 8-methoxypsoralen (8-MOP), 4,5′,8-trimethylpsoralen (TMP), and 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen (HMT) with a pair of 18-base-long oligonucleotides in which a 14-base region is complementary. Only one 5′TpA site, favored for both monoadduct and cross-link formation with psoralen, is present in this oligonucleotide pair. We have used this model system to demonstrate, for the first time, strand specificity in the photoreaction of psoralen with DNA. We found that the two types of cross-links which form at this site have large differences in thermal stabilities. In addition, the denaturation of each cross-link isomer duplex occurred in at least three stages, which can be visualized as three bands in thermal equilibrium under the conditions of a denaturing polyacrylamide gel. This novel observation suggests that there are several domains differing in thermal stability in a psoralen cross-link.

The cytotoxicity of psoralen plus long-wave UV light has been used for the treatment of skin ailments since ancient times (Scott et al., 1976). It is known that psoralen–DNA monoadducts and diadducts are mutagenic (Kirkland et al., 1983; Ashwood-Smith et al.; 1980; Bridges et al., 1979). The psoralen monoadduct and diadduct in cellular DNA are repaired by nucleotide excision repair systems, e.g., the UvrABC endonuclease system of *Escherichia coli* (Howard-Flanders, 1973). Because the chemical reactivity of psoralen with DNA is highly specific and controllable (Song & Tapley, 1979; Dall'Acqua et al., 1979) and because thymine–psoralen–thymine diadducts can be photoreversed (Shim & Kim, 1983; Thompson & Hearst, 1983; Kanne et al., 1982a) the psoralen–DNA diadducts are ideal substrates for the biochemical analyses of DNA repair at interstrand cross-links. The formation of a thymine–psoralen–thymine diadduct in a DNA duplex occurs in three steps. Psoralen first intercalates into the DNA helix in a noncovalent manner in one of two orientations (Figure 1). Either the 3,4-pyrone double bond or the 4′,5′-furan double bond of psoralen undergoes an addition reaction with the 5,6 double bond of a pyrimidine to form a pyrimidine–psoralen monoadduct in the cis–syn conformation (Kanne et al., 1982b; Peckler et al., 1982). Reaction of the 3,4-pyrone double bond destroys the coumarin nucleus of psoralen. However, if the 4′,5′-furan double bond is the first to react, the coumarin nucleus is intact and hence able to further absorb light at 365 nm and to react its 3,4-pyrone double bond with the 5,6 double bond of another pyrimidine to form a diadduct (Parsons, 1980; Kanne et al., 1982a). Thus, diadducts with the psoralen moiety in two opposite orientations can be formed, and we call these two types of cross-links "isomers". The pyrimidines involved in diadduct formation in DNA are usually thymine bases (Yeung et al., 1987; Tessman et al., 1985). The conformation of the isolated thymine–psoralen–thymine diadduct is cis–syn–cis (Kanne et al., 1982a), and the angle of distortion in the DNA helix is predicted as about 70° at each psoralen–DNA diadduct (Peckler et al., 1982). However, there is no information on the actual conformation of the psoralen cross-link inside a DNA helix or whether the two possible psoralen cross-links at a given cross-linkable site are structurally the same. 8-MOP,[1] TMP, and HMT are different derivatives of psoralen that are markedly different in their water solubility, affinity for DNA, and rates of photoreaction (Cimino et al., 1985).

In this study, we investigated whether there is significant difference in the cross-linking reactions of 8-MOP, TMP, and HMT. We tested whether the photoreversal reaction of the 8-MOP and TMP cross-links of our model DNA sequence occur on the furan side as well as the pyrone side. We examined whether the cross-link isomers at a given site are equivalent and investigated the effects of temperature on the denaturation of the cross-link isomers.

## Experimental Procedures

*Preparation of Oligonucleotides.* The 18-base oligonucleotides, named as in Figure 1, were synthesized by Applied Biosystems DNA synthesizer 380A and purified by FPLC (Pharmacia) anion-exchange chromatography on a Mono Q column and/or by fragment isolation on denaturing polyacrylamide gels. The DNA sequences of the oligonucleotides were verified by chemical DNA sequencing (Maxam & Gilbert, 1980).

*Electrophoresis of Oligonucleotides.* All polyacrylamide gels were denaturing gels, 0.5 mm × 30 cm × 40 cm, 20% acrylamide, 37.5:1 acrylamide to bis(acrylamide), 7 M urea, 1× TBE. All gels except the 63 °C gel were run between 43 and 49 °C at 2000 V for 3.5–4 h (until bromophenol blue was at the bottom of the gel). The tracking dye was 90% formamide, 0.1% xylene cyanole, and 0.25% bromophenol blue.

*Preparation of $^{32}$P-Labeled Oligonucleotides.* Oligos I and II were 5′ end-labeled by using γ-labeled [$^{32}$P]ATP and T4 polynucleotide kinase as described (Maxam & Gilbert, 1980) except unlabeled ATP was used to chase the phosphorylation reaction to completion. The labeled DNA was purified by electrophoresis on a 20% acrylamide, 7 M urea, DNA se-

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[1] Abbreviations: 8-MOP, 8-methoxypsoralen; TMP, 4,5′,8-trimethylpsoralen; HMT, 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; TBE, 50 mM Tris–borate and 1 mM EDTA, pH 8.3; oligo, oligonucleotide; FPLC, fast protein liquid chromatography.

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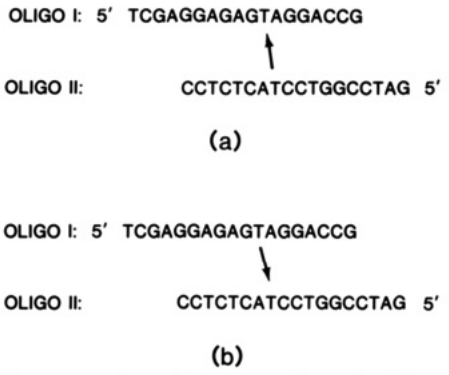


FIGURE 1: Sequences of complementary oligonucleotides used in this study. The arrows indicate the orientations of the psoralen molecule from the furan ring toward the pyrone ring. A 5′TpA sequence favoring the psoralen photoreaction is located in the middle of each oligonucleotide. In a psoralen photoreaction, either oligonucleotide may be first to react with psoralen, leading to two possibilities: in (a), the oligo II was first to react with the furan side of psoralen to form a monoadduct, then lead to the formation of the upper cross-link; in (b), the oligo I was first to react with the furan ring of the psoralen to form a monoadduct, then lead to the formation of the lower cross-link.

quencing gel, fragment isolated, and purified as described (Maxam & Gilbert, 1980).

*Photoreaction of TMP, 8-MOP, and HMT with the Oligonucleotides.* A typical photoreaction consists of 8 μg of each oligonucleotide (12.6 μM 5′TpA site), 5 μg/mL TMP (20 μM) or 25 μg/mL 8-MOP (106 μM), or 40 μg/mL HMT (140 μM) in 100 μL of 5 mM Tris-HCl, 0.2 mM EDTA, and 50 mM NaCl, pH 7.6. The conditions chosen are those commonly used in the literature for these psoralens. Long-wave UV dosage was determined by a UV Products J221 UV meter. UV light (365 nm) was provided by two Sylvania BT-28 bulbs mounted 10 cm apart fitted with aluminum parabolic reflectors and shone through a 1 cm 40% w/v cobalt nitrate liquid filter. The temperature of the reaction chamber was maintained at 25 °C by a refrigerated water jacket containing 1.5 mM adenosine and 0.02% sodium azide. Spectral analysis (not shown) of this filter setup indicated that the major transmission was between 330 and 430 nm and above 550 nm. Because the samples in these experiments are typically very small, in the range of 5–50 μL, the irradiation is usually done in microcentrifuge tubes instead of cuvettes (Gamper et al., 1984). Incidentally, the microcentrifuge tube blocks about 90–94% of all the 365-nm light. Thus, our effective dosage at the sample was 130 J $m^{-2}$ $s^{-1}$. Sarstedt 500-μL clear polypropylene microcentrifuge tubes were used throughout these experiments.

The reaction products were resolved on denaturing polyacrylamide gel and the monoadduct oligonucleotide and cross-linked oligonucleotides bands were visualized with autoradiography, extracted, and purified as described (Maxam & Gilbert, 1980). In general, the formation of the monoadducts and cross-links of 8-MOP were about 10 times slower than the reactions of TMP or HMT. We have performed experiments with both 40 and 5 μg/mL HMT so that our results could be compared with the experiments of Gamper et al. (1984), who used 8 times higher concentration of HMT than the 5 μg/mL in our TMP experiments. The gel-purified monoadduct-containing oligonucleotides were further reacted with the complementary strand to produce cross-linked oligonucleotides with the psoralen in the specified orientation. The isolated cross-links were used in 254-nm UV photoreversal experiments in which the DNA was irradiated by a 15-W

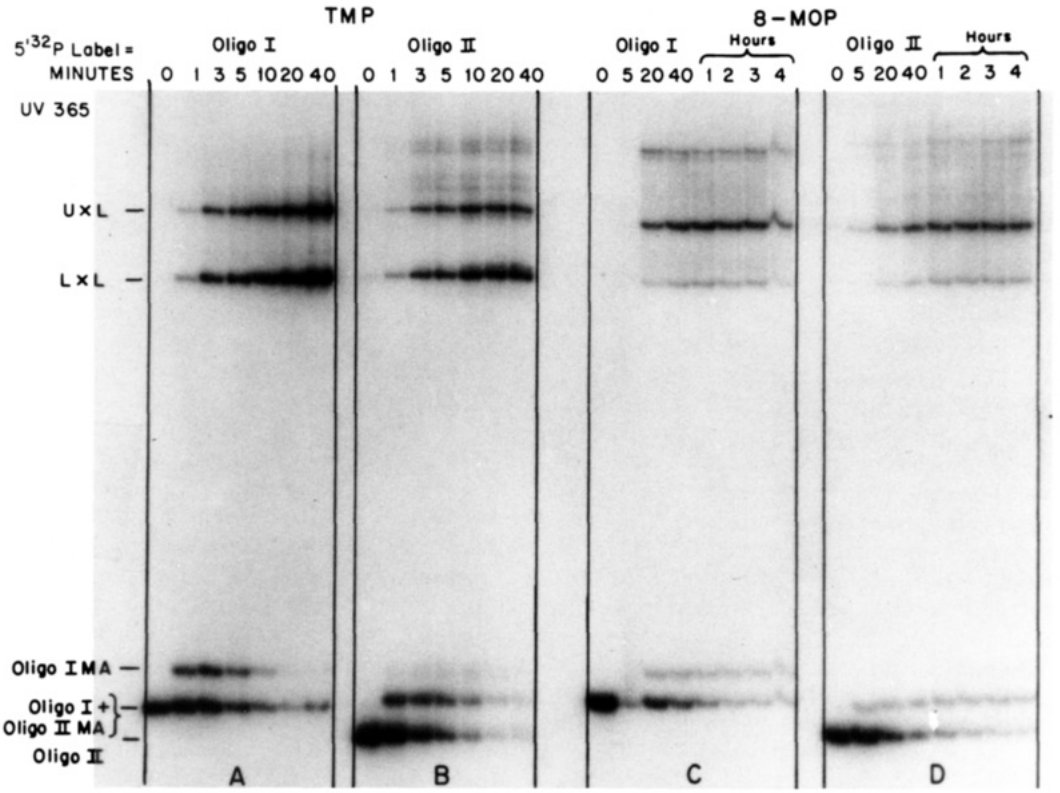


FIGURE 2: Formation of the monoadducts and cross-links of oligo I and oligo II in photoreactions with TMP and 8-MOP. The purified 5′-end-labeled fragments were used in the TMP (panels A and B) or 8-MOP (panels C and D) photoreactions. Oligo I was labeled in panels A and C, while oligo II was labeled in panels B and D so that the reactions on the two oligonucleotides can be visualized independently. The photoreaction conditions are as described under Experimental Procedures. At indicated time points, 5-μL samples were taken, dried, and washed with 0.5 mL of 95% ethanol. The samples were then resuspended in 3 μL of tracking dye, and 0.5-μL samples were applied to a DNA sequencing gel. Autoradiography was used to visualize the results of the DNA sequencing gel analysis. MA = monoadduct-containing oligonucleotide; UXL = upper cross-link; LXL = lower cross-link.

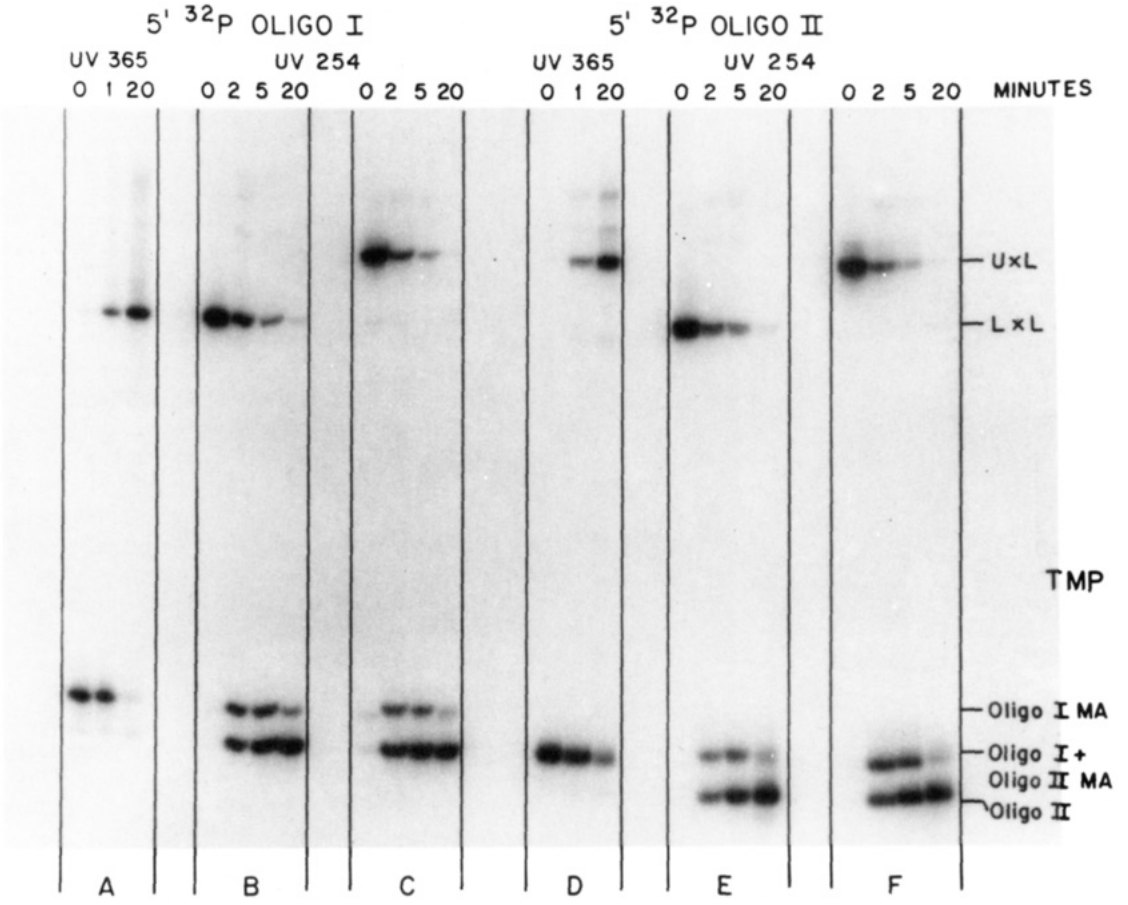


FIGURE 3: Photoreactions of the monoadducts and cross-links of TMP. A direct scale-up of the experiment in Figure 2 was performed to obtain sufficient quantities of each labeled monoadduct and cross-link of TMP shown in Figure 2, as described under Experimental Procedures. Oligo I was labeled in panels A–C, and oligo II was labeled in panels D–F. In panels A and D, the isolated monoadducts were hybridized to a slight excess of the unlabeled complementary strand and further irradiated at 365-nm UV light as described to form the cross-links. The 254-nm UV photoreversal reactions are shown in panels B, C, E, and F as described under Experimental Procedures. The lower cross-links are followed in panels B and E. The upper cross-links are followed in panels C and F. After irradiation, the samples were processed and analyzed as described in the legend to Figure 2. UXL = upper cross-link; LXL = lower cross-link.

General Electric germicidal lamp at a distance of 10 cm from the center of the lamp at a dosage of 33 J $m^{-2}$ $s^{-1}$ as measured by a UV Products J225 short-wave UV meter.

## Results and Discussion

*Strand Specificity in the Photoreactions of TMP and 8-MOP with the Oligonucleotides.* The photoreaction of TMP and 8-MOP with the oligonucleotides was reasonably rapid (Figure 2), with TMP reacting about 10 times faster than 8-MOP. For both psoralens, the formation of diadducts could be seen with the earliest appearance of the monoadducts. The presence of a psoralen monoadduct in an oligonucleotide causes the oligonucleotide to migrate in a denaturing polyacrylamide gel as though it is one base longer. This, the monoadduct of oligo I migrated one nucleotide spacing above oligo I. Due to base composition differences, oligo I migrated in the denaturing polyacrylamide gel as though it was one nucleotide longer than oligo II and comigrated with the monoadduct of oligo II. Two bands of cross-linked oligonucleotides were seen with the photoreaction of both psoralens, and we call the slower moving band and the faster moving band upper cross-link and lower cross-link, respectively, to reflect their relative positions in the vertically run gels. The relative amounts of the two bands of cross-link were evaluated by densitometer scanning of the autoradiogram (data not shown). The ratio of the upper cross-link to the lower cross-link was found to be about 3:2 for TMP whether oligo I or oligo II was 5′ phosphorylated. This ratio became about 10:1 in the case of 8-MOP. This is the first demonstration that the photoreactivity of psoralens at a given 5′TpA site may be different for the two complementary strands. Our present study examined the rates of formation of the orientational isomers at a 5′TpA site. Further, the site is not flanked by A's and T's but by G's and C's. Our study is different from previous studies that compared the photoreactivities of 5′TpA sites flanked by different A and T combinations and which did not compare the yields of the two isomers at each site (Yeung et al., 1987; Zhen et al., 1986; Sage & Moustacchi, 1987).

*Origin of Each Band of Psoralen Cross-Link.* To determine the origin of the two cross-linked bands, 5′ $^{32}$P-labeled oligonucleotides were photoreacted to produce monoadduct oligonucleotides at about 30% yield. These monoadduct oligonucleotides were gel purified and hybridized with a slight excess of the corresponding unlabeled complementary oligonucleotides and then irradiated with 365-nm UV light. Only one of the two oligonucleotides is labeled in each experiment so that only the reactions on that oligonucleotide will be visualized in the autoradiogram. While the experiment was performed with all three psoralens as shown in Figures 3, 5, and 6, for TMP, 8-MOP, and HMT, respectively, for the sake of simplicity, we will only discuss the data with TMP. It can be seen in Figure 3A that the monoadduct of oligo I had reacted to produce the lower cross-link band. In panel D the monoadduct of oligo II had reacted to produce the upper cross-link band. If a monoadduct-containing oligonucleotide can further react to form a cross-link, the initial reaction of

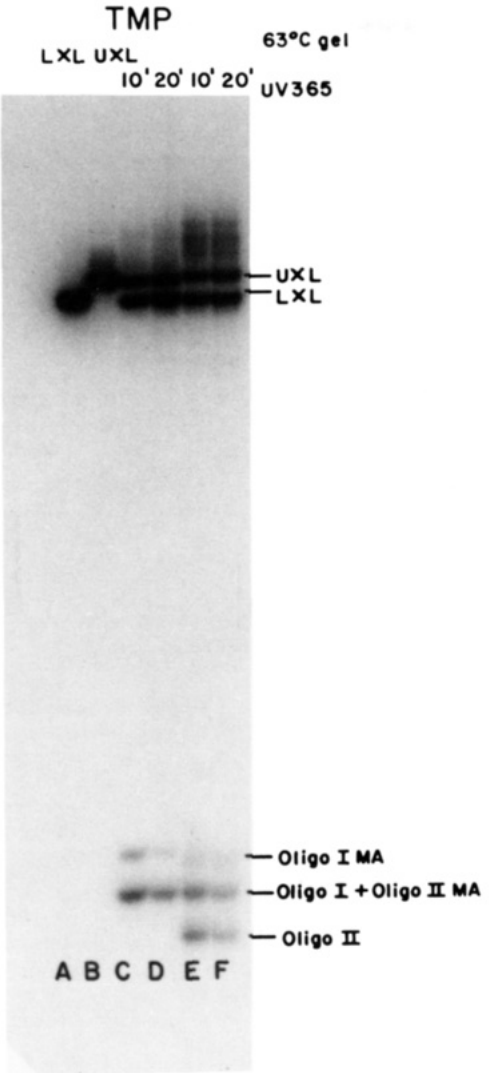


FIGURE 4: Change in the mobility of the TMP lower cross-link band during electrophoresis at 63 °C. Samples from the experiment in Figure 3 were analyzed on a denaturing polyacrylamide gel as the experiment in Figure 3 except that the gel temperature was maintained at 63 °C at all times. Lanes A and B are the gel-isolated lower cross-link and upper cross-link, respectively. In lanes C and D, the labeled oligo I and unlabeled oligo II were irradiated for 10 and 20 min, respectively, as for the experiment in panel A of Figure 2. In lanes E and F, the labeled oligo II and unlabeled oligo I were irradiated for 10 and 20 min, respectively, as for the experiment in panel B of Figure 2.

the psoralen in the formation of that monoadduct would necessarily have occurred at the furan side of the psoralen molecule so that the coumarin chromophore was not destroyed (Parsons, 1980; Kanne et al., 1982a). Thus, the upper cross-link had the furan side of the psoralens reacted with oligo II. The lower cross-link had the furan side of the psoralens reacted with oligo I. All the monoadducts isolated from the TMP photoreaction appeared to be the furan-side monoadducts since all of them could further react to form diadducts (Figure 3A). This is in agreement with the known chemical reactivity of TMP (Song & Tapley, 1979). The 8-MOP monoadducts that have accumulated, however, appeared to be mostly the pyrone-side monoadducts since only a small percentage of them could further photoreact to form diadducts (Figure 5A,D). This is consistent with the observation that 8-MOP photoreaction with DNA results in about 20% of the 8-MOP adduct accumulating as the pyrone-side monoadducts (Tessman et al., 1985). The similar rates of formation of cross-links from the isolated monoadducts in the experiment in Figure 5A,D suggest that the observed strand specificity for the formation of 8-MOP cross-link is the result of the different rates of formation of the furan-side 8-MOP monoadduct in each DNA strand and not due to difference in the rate of conversion of each monoadduct to the respective cross-links. In Figure 2, for both 8-MOP and TMP, two pairs of bands were seen above the two major cross-link bands in each lane. These bands, which have slower mobility than the major cross-link bands, represent other conformations of the same cross-linked oligonucleotides in equilibrium with the two major cross-link bands. Their significance will be discussed further in a later section.

*Photoreversal of the TMP and 8-MOP Cross-Links Occurs on both the Furan Side and the Pyrone Side.* Interstrand cross-links of psoralens can be photoreversed by 254-nm UV light (Shim & Kim, 1983; Thompson & Hearst, 1983; Kanne et al., 1982a; Gamper et al., 1984; Cimino et al., 1986; Shi & Hearst, 1987a; Van Houten et al., 1986). We tested whether photoreversal will occur on both the furan and the pyrone side of the cross-links in our model oligonucleotide for all three psoralen derivatives. In Figure 3, the upper cross-link and the lower cross-link oligonucleotides from TMP reactions were resolved and isolated from denaturing polyacrylamide gels. When these cross-links were irradiated by 254-nm UV light, all of them were photoreversed at about the same rate with the formation of the corresponding monoadduct-containing oligonucleotides, followed by conversion to the psoralen-free oligonucleotides (panels B, C, E, and F of Figure 3). For example, in Figure 3, oligo I was labeled to test the lower cross-link in panel B and to test the upper cross-link in panel C. Upon irradiation by 4000 J/m$^2$ 254-nm UV light, the formation of monoadduct-containing oligo I can be seen. Since the TMP was bonded to oligo I via the furan side in lower cross-link and via the pyrone side in upper cross-link, the appearance of monoadduct-containing oligo I in both panels B and C demonstrated photoreversal at the pyrone side and the furan side in panels B and C, respectively. Thus, we conclude that the furan- and pyrone-side cyclobutane rings of the TMP cross-link are equally likely to be reversed by 254-nm UV light. Upon further irradiation to 40 000 J/m$^2$, all the oligonucleotides returned to the psoralen-free form. Identical results were observed in the experiments in panels E and F of Figure 4 in which the oligo II was 5′ phosphorylated with $^{32}$P instead of oligo I so that the reactions of oligo II were visualized. Thus, it is clear that photoreversal of TMP cross-links occur on both the furan and the pyrone sides of TMP, regardless of whether the 5′ end of the oligonucleotides were phosphorylated and regardless of the orientation of the psoralen molecule between the two oligonucleotides. Similar results were obtained for the photoreversal of the cross-link isomers of 8-MOP (Figure 5) and HMT (Figure 6).

*Psoralen Cross-Link Isomers Can Have Large Differences in Thermal Stabilities.* The dramatic difference in the mobilities of the two psoralen cross-links isomers has demonstrated for the first time that cross-link isomers at the same site may have very different properties. The cross-link isomers used in previous studies all tend to show almost identical gel mobilities (Van Houten et al., 1986; Shi & Hearst, 1986, 1987a,b). The different resolutions may be due to the nature of the DNA sequences used. A cross-link can stabilize a helix from thermal melting (Shi & Hearst, 1986). However, it is not known if the two orientational isomers of psoralen cross-link have different thermal stabilities. We predict that of the two cross-link isomers we observe the lower cross-link is of a more stable helical structure and that it will remain

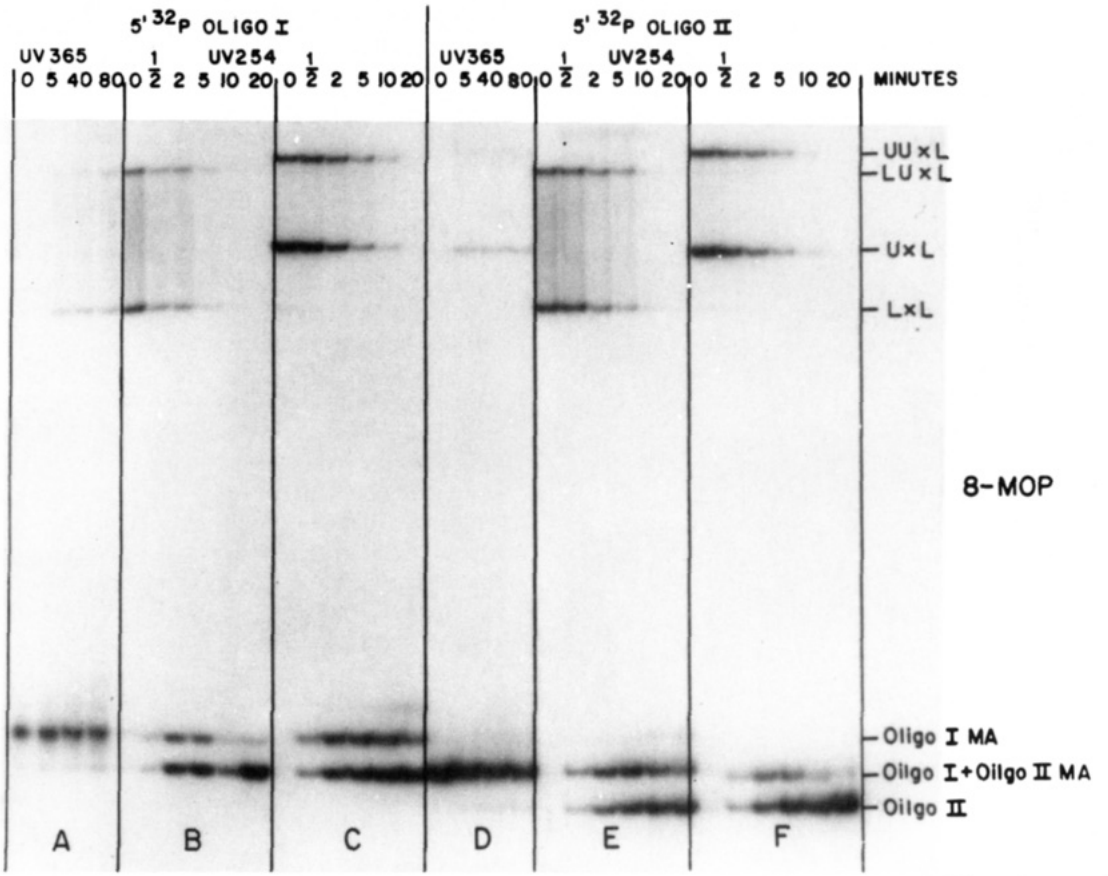


FIGURE 5: Photoreactions of the monoadducts and cross-links of 8-MOP. A direct scale-up of the experiment in Figure 2 was performed to obtain sufficient quantities of each labeled monoadduct and cross-link of 8-MOP shown in Figure 2, as described under Experimental Procedures. Oligo I was labeled in panels A–C, and oligo II was labeled in panels D–F. In panels A and D, the isolated monoadducts were hybridized to a slight excess of the unlabeled complementary strand and further irradiated at 365-nm UV light as described to form the cross-links. The 254-nm UV photoreversal reactions are shown in panels B, C, E, and F as described under Experimental Procedures. The lower cross-links are followed in panels B and E. The upper cross-links are followed in panels C and F. After irradiation, the samples were processed and analyzed as described in the legend to Figure 2. Notice the splitting of the upper cross-link band (UXL) to produce the upper–upper cross-link band (UUXL) and the lower cross-link band (LXL) splitting to produce the lower–upper cross-link (LUXL) in this electrophoresis. It is easier to visualize the relative positions of the various cross-link bands by sighting down the figure from its right side.

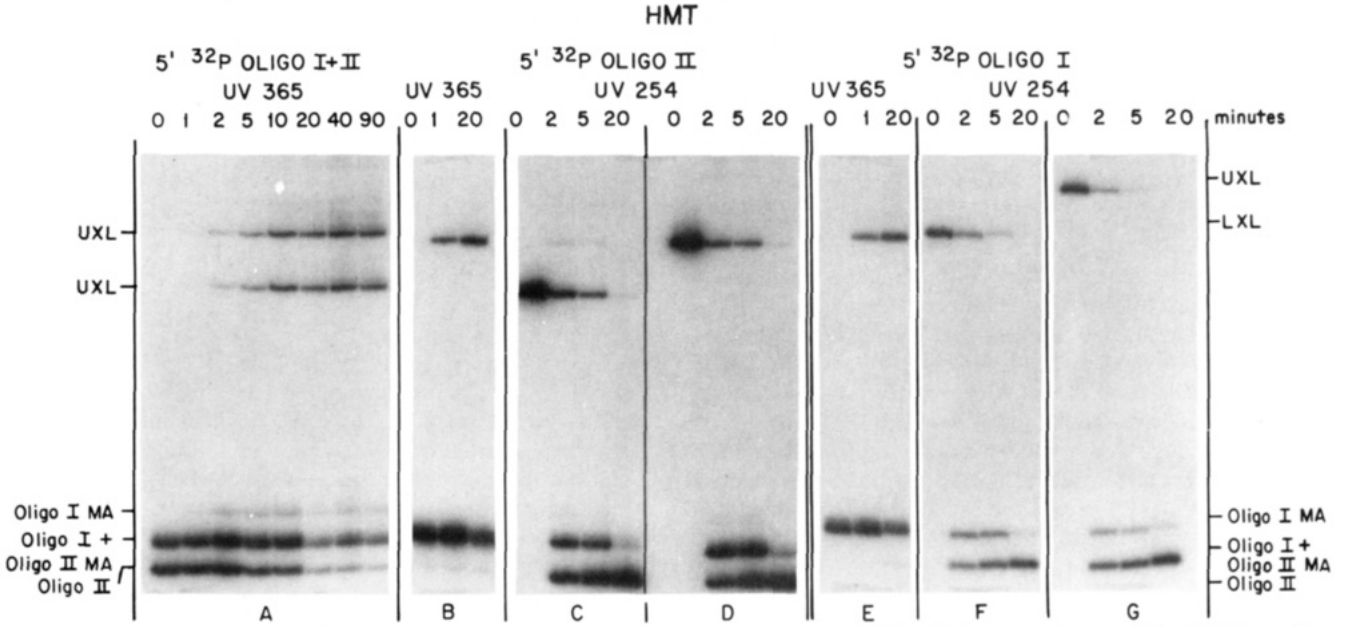


FIGURE 6: Rate of formation and photoreversal of HMT cross-links. The HMT was at 40 μg/mL in all these experiments. (Panel A) Formation of monoadduct and cross-links of oligo I and II in photoreaction with HMT. The experiment was identical with that in Figure 2 for TMP except that both oligonucleotides were 5′ $^{32}$P-labeled. Only oligo II was $^{32}$P-labeled in panels B–D. Only oligo I was $^{32}$P-labeled in panels E–G. (Panels B, E) Formation of upper cross-link from HMT monoadduct on oligo II and lower cross-link from HMT monoadduct on oligo I, respectively. The experiment was identical with that for TMP in Figure 3, panels A and D, respectively. Since panels A–D were run on one gel and panels E–G on another, the positions of the bands in these panels do not line up. (Panels C, F) Photoreversal of HMT lower cross-link to monoadduct and unmodified DNA. (Panels D, G) Photoreversal of upper cross-link of HMT to monoadduct and unmodified DNA.

more compact even under the denaturing conditions of the DNA sequencing gel. In other words, it is less melted even in 7 M urea at 49 °C in 1 × TBE. To test this hypothesis, the gel was maintained at temperatures higher than 49 °C in several experiments. Figure 4 represents the results of electrophoresis of TMP reacted oligonucleotides at 63 °C. By measuring the relative mobilities of the upper cross-link band and the lower cross-link bands at both 49 and 63 °C, with respect to the unreacted oligo II band (data not shown), it became apparent that the lower cross-link band had decreased its mobility at 63 °C such that its mobility is now similar to that of the upper cross-link band. The cross-linked oligonucleotide duplex has higher stability when the furan side of the psoralen is bonded to oligo I. Thus, we have demonstrated for the first time that the orientational isomers of psoralen cross-link have large differences in thermal stabilities. Since this work suggests that a psoralen cross-link at a given site can produce different DNA conformations, we have tested if they are repaired differently by the nucleotide excision system of *E. coli*. Our studies revealed that the UvrABC endonuclease incises the lower cross-link about 10 times faster than the upper cross-link when each cross-link is located in the middle of a 57-base-long oligonucleotide pair (unpublished data).

*A Psoralen Cross-Link Isomer Duplex Has Several Domains of Different Thermal Stabilities.* In Figure 2, several fainter bands were visible in the gel above the upper cross-link bands, and their origin was at first a puzzle. It turned out that even the upper cross-link does not represent the fully melted conformation of the cross-linked oligonucleotides. Although the gels in Figures 2 and 4 were performed under identical conditions, 8-MOP cross-links were found to be less stable than the TMP cross-links, and small differences in the conditions of the gel may have caused the cross-links to be melted more in the gel in Figure 5 than in the gel in Figure 2B. It can be seen in Figure 5B,C,E,F that when the gel-purified upper or lower cross-link bands of 8-MOP reaction was analyzed by another denaturing polyacrylamide gel, a new pair of slower moving cross-link bands appeared, which we shall call upper–upper cross-link (UUXL) and lower–upper cross-links (LUXL), respectively. The upper–upper cross-link in Figure 5 had come from the gel-isolated upper cross-link band, while the lower–upper cross-link had come from the gel-isolated lower cross-link bands. It appears from the equilibrium of the upper and lower sets of cross-links at 49 °C in 7 M urea that the psoralen cross-link produces domains of discrete stabilities in the DNA helix such that there are at least two melting temperatures for each cross-link isomer. It will be interesting to examine which regions of the cross-linked oligonucleotides constitute which level of stability and how these domains affect the biological properties of a psoralen cross-link. We would like to define what features in this DNA sequence are responsible for the strand specificity of 8-MOP.

*Photoreactions of HMT with the Oligonucleotides.* We found that HMT reacted, at our UV dosage, only slightly faster at 40 μg/mL than at 5 μg/mL but was otherwise identical in photoreactivity (data not shown), suggesting little need for the use of the higher HMT concentration. The forward reaction of HMT with 5′-labeled oligo I and II is of similar rate (Figure 6A) if not slightly slower than the reaction of TMP (Figure 2A). We used gel-purified upper cross-link or lower cross-link from HMT-reacted oligo I and II in which only one strand is labeled and repeated the photoreversal experiments described above for TMP and 8-MOP. We found that HMT cross-links were also photoreversed by 254-nm UV light randomly both on the furan side and on the pyrone side, with no apparent sequence specificity (Figure 6C,D,F,G). Reactivity of HMT with bases other than the 5′TpA sequence is probably responsible for the persistence of HMT monoadducts that cannot continue to form cross-links (Figure 6B,E). This reactivity of HMT with sequences other than 5′TpA, 5′ApT, and 5′TpG was also detected when the positions of HMT monoadducts in restriction fragments of natural DNA were diagnosed with the incision by the UvrABC endonuclease (Sancar et al., 1985).

In summary, we have shown that the two possible psoralen cross-links at a given cross-linkable site are not equivalent. We found that the two cross-link isomers formed at this site have large differences in thermal stability. We observed that there are several domains of different thermal stabilities in a psoralen cross-link. TMP is more specific than 8-MOP and HMT for the formation of monoadduct and cross-link in the 5′TpA sequence used in this study. 8-MOP showed a preference for one of the two DNA strands for initiating the formation of DNA cross-link in this model system. For all three psoralens, the photoreversal, by 254-nm UV light, of the cyclobutane rings, linking the thymine moieties with either the furan or the pyrone ring of the psoralen molecule, was found to occur at about the same efficiency on both the furan and the pyrone side of the psoralen molecule.

**Registry No.** 8-MOP, 298-81-7; TMP, 3902-71-4; HMT, 62442-59-5; oligo I–oligo II duplex, 113474-56-9; psoralen, 66-97-7.

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# Efficient Synthesis of a Supercoiled M13 DNA Molecule Containing a Site Specifically Placed Psoralen Adduct and Its Use as a Substrate for DNA Replication†

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ABSTRACT: We report a simple method for the in vitro synthesis of large quantities of site specifically modified DNA. The protocol involves extension of an oligonucleotide primer annealed to M13 single-stranded DNA using part of the T4 DNA polymerase holoenzyme. The resulting nicked double-stranded circles are ligated and supercoiled in the same tube, producing good yields of form I DNA. When the oligonucleotide primer is chemically modified, the resultant product contains a site-specific lesion. In this study, we report the synthesis of an M13 mp19 form I DNA which contains a psoralen monoadduct or cross-link at the *Kpn*I site. We demonstrate the utility of these modified substrates by assessing the ability of the bacteriophage T4 DNA replication complex to bypass the damage and show that the psoralen monoadduct poses a severe block to the holoenzyme when attached to the template strand.

There is considerable interest in the mechanism by which various structural modifications of DNA influence aspects of nucleic acid metabolism such as replication, transcription, and repair. These include lesions due to radiation, chemical carcinogens, and certain drugs. In vitro enzymatic studies have been hampered by the difficulty of producing workable quantities of DNA modified at a unique position. Such substrates would allow far more detailed questions to be asked and would yield more easily interpretable results than experiments using randomly modified nucleic acids. We report here a simple method for the in vitro synthesis of large quantities of supercoiled M13 double-stranded DNA containing a uniquely placed psoralen monoadduct or cross-link. The method is general and should be amendable to the synthesis of substrates with many other types of structural modifications.

Psoralens are a class of linear, tricyclic, aromatic molecules which readily intercalate into double-stranded nucleic acid. In the presence of near-ultraviolet light, these compounds react to form monoadducts and interstrand cross-links primarily with thymidine in DNA and with uridine in RNA [reviewed by Cimino et al. (1985)]. This activity has made them useful as chemotherapeutic agents in the treatment of several dermatological disorders including psoriasis and vitilago [for review, see Fitzpatrick et al. (1982) and Parrish et al. (1982)]. As part of an effort to better understand the biological consequences of these compounds, we have investigated the ability of the "core" T4 replication complex to synthesize past a psoralen monoadduct using a site specifically modified M13 substrate. We found the synthesis, which was initiated from a unique strand-specific nick, to be blocked by the monoadduct.

## Materials and Methods

*Preparation of Proteins and DNA.* The phage T4 43, 44/62, and 45 proteins were purified by using the protocols developed by W. Konigsberg and co-workers, Yale University (personal communication). T4 DNA ligase was purchased from Bethesda Research Laboratories. DNA gyrase was a gift from N. Cozzarelli. Phage fd gene II protein was purified by the literature procedure (Dotto et al., 1981). M13 mp 19 single-stranded and double-stranded DNAs were prepared by the procedures described by Messing (1983).

*Preparation of 4-(Hydroxymethyl)-4,5′,8-trimethylpsoralen (HMT)-Monoadducted 13-mer.* The 13-mer 5′-GCTCGGTACCCGG-3′ was synthesized on a Biosearch instrument using phosphotriester chemistry. Following deprotection, full-length product was isolated by electrophoesis on a 7 M urea–20% polyacrylamide gel. The desired band was detected by placing the gel over a thin-layer chromatography plate impregnated with a fluorophore and briefly illuminating in the dark with a shortwave ultraviolet lamp. After the band was excised, the 13-mer was extracted into 10 mM NaCl–1 mM ethylenediaminetetraacetic acid (EDTA), ethanol precipitated, and dissolved in 10 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl)–1 mM EDTA (TE).

Photochemical modification of the 13-mer with 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen (HMT) was carried out by using the two-step procedure described by Van Houten et al. (1986) and Gamper et al. (1987) or, more recently, by a simpler one-step procedure. Using the latter procedure, we obtained preparative amounts of psoralen-monoadducted ol-

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