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Interaction of Psoralen-Derivatized Oligodeoxyribonucleoside Methylphosphonates with Single-Stranded DNA[†]

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ABSTRACT: Oligodeoxyribonucleoside methylphosphonates derivatized at the 5′ end with 4′-(aminoalkyl)-4,5′,8-trimethylpsoralen were prepared. The interaction of these psoralen-derivatized methylphosphonate oligomers with synthetic single-stranded DNAs 35 nucleotides in length was studied. Irradiation of a solution containing the 35-mer and its complementary methylphosphonate oligomer at 365 nm gave a cross-linked duplex produced by cycloaddition between the psoralen pyrone ring of the derivatized methylphosphonate oligomer and a thymine base of the DNA. Photoadduct formation could be reversed by irradiation at 254 nm. The rate and extent of cross-linking were dependent upon the length of the aminoalkyl linker between the trimethylpsoralen group and the 5′ end of the methylphosphonate oligomer. Methylphosphonate oligomers derivatized with 4′-[[*N*-(2-aminoethyl)amino]methyl]-4,5′,8-trimethylpsoralen gave between 70% and 85% cross-linked product when irradiated for 20 min at 4 °C. Further irradiation did not increase cross-linking, and preirradiation of the psoralen-derivatized methylphosphonate oligomer at 365 nm reduced or prevented cross-linking. These results suggest that the methylphosphonate oligomers undergo both cross-linking and deactivation reactions when irradiated at 365 nm. The extent of cross-linking increased up to 10 μM oligomer concentration and dramatically decreased at temperatures above the estimated T_m of the methylphosphonate oligomer–DNA duplex. The cross-linking reaction was dependent upon the fidelity of base-pairing interactions between the methylphosphonate oligomers and the single-stranded DNA. Noncomplementary oligomers did not cross-link, and the extent of cross-linking of oligomers containing varying numbers of noncomplementary bases was greatly diminished or eliminated. The extent and sequence specificity of photoinduced cross-linking, combined with the known ability of methylphosphonate oligomers to be taken up by living cells, suggest that psoralen-derivatized oligonucleoside methylphosphonates may be useful probes of cellular gene expression.

Recent reports from a number of laboratories have demonstrated that oligonucleotides and oligonucleotide analogues whose sequences are complementary to target mRNA are capable of inhibiting translation of mRNA in cell-free systems and can prevent mRNA expression in various cell culture systems (Zamecnik & Stephenson, 1978; Stephenson & Zamecnik, 1978; Blake et al., 1985a; Kawasaki, 1985; Zamecnik et al., 1986; Cazenave et al., 1986, 1987; Toulme et al., 1986; Lawson et al., 1986; Haeuptle et al., 1986; Wickstrom et al., 1986; Lemaitre et al., 1987; Gupta, 1987; Neckers et al., 1987; Maher & Dolnick, 1987; Marcus-Sekura et al., 1987). Our laboratory has demonstrated that oligodeoxyribonucleoside methylphosphonates are able to inhibit the expression of mRNA both in cell-free translation systems and in virus-infected cells. For example, methylphosphonate oligomers whose sequences are complementary to the initiation codon regions of rabbit globin mRNA inhibit translation in rabbit reticulocyte lysates and in rabbit reticulocytes (Blake et al., 1985b). Oligomers complementary to the initiation codon regions of vesicular stomatitis virus (VSV) N, NS, or G protein mRNAs were found to inhibit VSV mRNA translation in a reticulocyte lysate system and to specifically inhibit virus protein synthesis in VSV-infected mouse L cells (Agris et al., 1986). In another study, Smith et al. (1986) demonstrated that a methylphosphonate oligomer complementary to the acceptor splice junctions of Herpes simplex virus type I (HSV-1) immediate early precursor mRNA 4 and 5 inhibited virus protein synthesis, virus DNA synthesis, and virus growth in HSV-1-infected Vero cells. As was the case for VSV, the methylphosphonate oligomer specifically inhibited virus function and did not affect cellular protein synthesis or growth. On the basis of the results of these studies, it appears that oligonucleotides and oligonucleoside methylphosphonates hold considerable potential for studying and perhaps controlling viral and cellular gene expression in cell culture systems.

It is generally believed that oligonucleotides or oligonucleotide analogues exert their inhibitory effects by a hybridization arrest type mechanism. That is, the oligomer binds to its complementary sequence on the mRNA and prevents, for example, translation of the message by preventing ribosome binding. In some cases, it has been demonstrated that the oligomer activates ribonuclease H catalyzed cleavage of the mRNA as a consequence of forming a duplex with the mRNA (Minshull & Hunt, 1986). This mode of inhibition appears to occur in wheat germ extracts, in oocytes, and possibly in mammalian cells. RNase H activity appears to be absent in rabbit reticulocyte lysates (Lawson et al., 1986; Cazenave et al., 1987).

Although the evidence for specific interaction between oligonucleotides and their target mRNAs in cell-free systems is quite strong, it is more difficult to assess the mechanism of action of the oligomers in cell culture systems. It has not been possible to isolate complexes between the oligomer and its target mRNA. Similarly, it is not possible to rule out that the oligomer is binding to other nucleic acids within the cell.

If oligonucleotides are to be used as mechanistic tools to probe and study gene expression in biological systems, it is

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important that the binding site of the oligomer be known with certainty. It should be possible to achieve this goal if the oligomer is modified in such a way that it can covalently cross-link with its target nucleic acid. Such a derivatized oligomer would have the added potential advantage that it could function at lower concentrations than nonderivatized oligomers, which rely solely on hydrogen-bonding interactions for their inhibitory activity. Toward this end, we have prepared oligonucleoside methylphosphonates derivatized with 4′-(aminoalkyl)-4,5′,8-trimethylpsoralen photoactivatable cross-linking groups. In this paper, we describe the synthesis and properties of these oligonucleotide analogues and studies on their interaction with synthetic single-stranded DNAs.

EXPERIMENTAL PROCEDURES

[γ-^{32}P]ATP was purchased from Amersham Inc. T4 polynucleotide kinase was purchased from Bethesda Research Laboratories, and 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide was obtained from Sigma Chemicals. SEP-PAK C-18 reverse-phase cartridges were purchased from Waters Associates. Polyacrylamide gel electrophoresis was carried out on 20 cm × 20 cm × 0.75 mm gels containing 15% acrylamide and 7 M urea. The gels were run in TBE buffer which contained 0.089 M tris(hydroxymethyl)aminomethane (Tris), 0.089 M boric acid, and 0.2 mM ethylenediaminetetraacetic acid (EDTA) (Maniatis et al., 1982).

Synthesis of 4′-(Aminoalkyl)-4,5′,8-trimethylpsoralens. 4′-(Aminomethyl)-4,5′,8-trimethylpsoralen (AMT) was prepared according to the procedure of Isaacs et al. (1977). 4′-[[*N*-(2-Aminoethyl)amino]methyl]-4,5′,8-trimethylpsoralen [(ae)AMT] and 4′-[[*N*-(4-aminobutyl)amino]methyl]-4,5′,8-trimethylpsoralen [(ab)AMT] were synthesized as follows. To a suspension of 0.154 mmol of 4′-(chloromethyl)-4,5′,8-trimethylpsoralen (Isaacs et al., 1977) in 2.5 mL of acetonitrile was added a solution of 1.54 mmol of ethylenediamine or 1,4-diaminobutane dissolved in 1 mL of acetonitrile. The reaction mixture was stirred 1 h and an additional 1-mL portion of the 1.54 M diaminoalkane solution was added. The reaction was followed by thin-layer chromatography (TLC) on silica gel using 10% methanol in chloroform as the solvent. The reaction was over after 6 h as indicated by the disappearance of the starting material, whose R_f was 0.74, and the appearance of the product, whose R_f was 0.04. In the case of (ae)AMT, an additional spot at R_f 0.19 was also observed. The reaction mixture was diluted with 30 mL of chloroform and extracted with four 5-mL portions of water. The chloroform solution was dried over anhydrous sodium sulfate and, after filtration, evaporated to give an oily residue. The oil was taken up in 4 mL of water, and the pH of the cloudy solution was adjusted to 7 by dropwise addition of 1 M HCl. In the case of (ae)AMT, acetonitrile was added until the solution became clear. The product crystallized after several days at 4 °C. The crystals were collected, washed with ice-cold water, and dried under vacuum. Alternatively, the residue obtained from the chloroform extract was taken up in 2 mL of water and titrated with acetonitrile until a clear solution was obtained. This solution was loaded onto a C-18 reversed-phase column, and the product was eluted with a linear gradient of 50–80% acetonitrile in water. The product was recovered by evaporation of the solvents followed by lyophilization from water. In the case of (ab)AMT, the aqueous solution was diluted with 6 mL of water, and the clear solution was passed through a SEP-PAK cartridge. The SEP-PAK was washed with five 10-mL portions of 25 mM triethylammonium bicarbonate (TEAB), and the product was eluted with 3 mL of 50% acetonitrile in 100 mM TEAB. The buffer was evaporated on a Speed Vac, and the residue was lyophilized from water. The yield of both products was approximately 0.05 mmol. The ultraviolet spectra of both compounds in water were virtually identical with that of AMT with λ_{max} at 247, 297, and sh 335 nm and λ_{min} at 226 and 258 nm. The proton NMR spectra in deuteriochloroform were consistent with the proposed structure. For (ae)AMT, the following resonances were observed: three singlets at 2.198, 2.379, and 2.582 ppm (three CH_3–); two multiplets at 3.359 and 3.559 ppm (–CH_2CH_2–); a singlet at 4.461 ppm (4′-CH_2–); a singlet at 6.125 ppm (3-H); and a singlet at 7.631 ppm (5-H). The solid compounds were stored in the dark at room temperature. For use in synthetic reactions, a 0.05 M solution was prepared by suspending the appropriate amount of compound in 100 μL of water and adding 1-μL aliquots of 1 M hydrochloric acid until a clear solution was obtained.

Synthesis of Oligodeoxyribonucleotides. Oligodeoxyribonucleotides **1** and **2** (Figure 2) were synthesized by the β-(cyanoethyl)phosphoramidite method (Sinha et al., 1983) on controlled pore glass supports using a Systec Model 1440 DNA synthesizer. After cleavage from the support and deprotection, the crude 35-mers were phosphorylated with [^{32}P]ATP by polynucleotide kinase (Murakami et al., 1985). The phosphorylated 35-mers were purified by gel electrophoresis, desalted (Lo et al., 1984), and sequenced by the procedure of Chandrasegaran et al. (1985). The ^{32}P-labeled, gel-purified 35-mers were used in the cross-linking experiments described below.

Synthesis of Oligodeoxyribonucleoside Methylphosphonates. The methylphosphonate oligomers were synthesized on a 1% cross-linked polystyrene support using 5′-(dimethoxytrityl)nucleoside 3′-(methylphosphonic imidazolide) intermediates (Miller et al., 1986) or on a controlled-pore glass support using 5′-(dimethoxytrityl)nucleoside 3′-[[(*N*,*N*-diisopropylamino)methyl]phosphonamidite] intermediates (Dorman et al., 1984; Jager & Engels, 1984; Marcus-Sekura et al., 1987) which were purchased from American Bionetics Inc. To suppress formation of 4-(2-aminoethyl)cytosine during the deblocking procedure (Miller et al., 1986), the support was treated overnight with a solution containing 1.2 mL of pyridine, 0.3 mL of glacial acetic acid, and 0.05 mL of 85% hydrazine hydrate to remove benzoyl groups from cytosine and adenine residues of the protected oligomer (Letsinger et al., 1968). The partially deprotected oligomer was then further deprotected and cleaved from its support by sequential treatment with 50% ethylenediamine in ethanol and with 80% acetic acid. The oligomer was purified by DEAE-cellulose chromatography (Miller et al., 1986) and then further purified on a SEP-PAK C-18 cartridge. The SEP-PAK was first washed with 10 mL of 25 mM TEAB and 5 mL each of 5%, 10%, and 15% acetonitrile in 25 mM TEAB to remove buffer and any shorter oligomers. The product was eluted with 3 mL of 50% acetonitrile in 100 mM TEAB. The buffer was removed by evaporation, and the oligomer was lyophilized from water. The oligomer was phosphorylated and sequenced as previously described (Murakami et al., 1985; Miller et al., 1986).

Synthesis of 4′-(Aminoalkyl)-4,5′,8-trimethylpsoralen Derivatives of Oligodeoxyribonucleoside Methylphosphonates. The 5′-phosphorylated methylphosphonate oligomer was dissolved in 40 μL of 0.75 M lutidine hydrochloride buffer (pH 7.5). A solution of 0.05 M 4′-(aminoalkyl)-4,5′,8-trimethylpsoralen in water (5 μL) and a solution of 1 M 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (5 μL) were added. In cases where a cloudy solution formed, sufficient acetonitrile was added to clarify the solution. The reaction mixture was incubated at room temperature overnight and then diluted with water. The 4′-(aminoalkyl)-4,5′,8-trimethylpsoralen-derivatized oligomer was purified by DEAE-cellulose column chromatography using a linear gradient of 0.0–0.5 M ammonium bicarbonate. The derivatized methylphosphonate oligomer, which eluted before the unreacted phosphorylated oligomer, was desalted on a SEP-PAK cartridge as described above, and the buffer was removed by evaporation. The isolated yields of the derivatized oligomer were generally 85% or greater. Each oligomer was stored as a solution in 25% ethanol at −20 °C.

^{32}P-Labeled 4′-(aminoalkyl)-4,5′,8-trimethylpsoralen-derivatized oligonucleoside methylphosphonates were prepared in a manner similar to that described above, starting from the 5′-^{32}P-phosphorylated oligomer, and were purified by polyacrylamide gel electrophoresis.

Synthesis of the 4′-(Aminomethyl)-4,5′,8-trimethylpsoralen Derivative of Thymidine 5′-Phosphate (d-AMTpT). A solution of thymidine 5′-phosphate (62 μmol) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (620 μmol) in 0.68 mL of 0.1 M imidazole hydrochloride buffer (pH 6.0) was stirred at room temperature for 1 h. 4′-(Aminomethyl)-4,5′,8-trimethylpsoralen (68 μmol) dissolved in 2.3 mL of 0.5 M lutidine hydrochloride buffer (pH 7.5) was added, and stirring was continued in the dark at room temperature. The formation of the d-AMTpT was followed by reversed-phase high-performance liquid chromatography (HPLC) on a 25-cm Whatman ODS-3, 10-μm column using a linear gradient of 0.5–35% acetonitrile in 0.1 M ammonium acetate buffer (pH 5.8) at a flow rate of 2.5 mL/min. The retention times of d-pT, d-AMTpT, and AMT were 5.5, 22.1, and 24 min, respectively. The conversion of d-pT to d-AMTpT was 64% after 72 h. Lutidine was removed from the reaction by evaporation, and the d-AMTpT was purified by DEAE-cellulose column chromatography. The column was washed with water and 170 mL of 0.02 M ammonium bicarbonate. The product was eluted with 30 mL of 0.2 M ammonium bicarbonate and was recovered by evaporation of the buffer followed by lyophilization from water. The UV spectrum of the compound gave λ_{max} 252, sh 304, and 343 nm and λ_{min} 230 and 290 nm. The proton NMR spectrum of the compound in ^2H_2O was consistent with its structure and showed resonances at 1.50 (5-CH_3, T), 1.39 and 2.05 (H′2 and H″2, T), 3.79 (H′5, H′5, T), 4.00 (H′4, T), 7.10 (H6, T), 4.18 (H′3, T), 5.84 (H′1, T), 2.16, 2.32, and 2.40 (CH_3–, AMT), 3.95 (–CH_2–, AMT), 6.03 (H3, AMT), and 7.37 ppm (H5, AMT). The ratio of d-pT to AMT was 1:1 as determined by NMR spectroscopy. Treatment of d-AMTpT with 0.01 M hydrochloric acid at 37 °C for 4 h yielded d-pT and AMT as shown by reversed-phase HPLC.

Interaction of 4′-(Aminoalkyl)-4,5′,8-trimethylpsoralen-Derivatized Oligodeoxyribonucleoside Methylphosphonates with Oligodeoxyribonucleotides **1** *and* **2**. A solution of 0.15 μM ^{32}P-labeled 35-mer **1** or **2** (see Figure 2) and 10 μM 4′-(aminoalkyl)-4,5′,8-trimethylpsoralen-derivatized oligonucleoside methylphosphonate in 5 μL of buffer containing 10 mM Tris and 0.1 mM EDTA at pH 7.5 was preincubated in a borosilicate glass test tube at 37 °C for 5 min. The solution was then irradiated at 365 nm for 0–60 min at an intensity of 0.83 J/cm^{-2} min^{-1} in a thermostated water bath using a long-wavelength ultraviolet lamp (Ultra-Violet Products Inc.). The borosilicate glass absorbed light below 300 nm. The reaction mixture was lyophilized, dissolved in gel loading buffer, and subjected to gel electrophoresis on a 15% polyacrylamide gel containing 7 M urea. For photoreversal experiments, the reaction mixture obtained after irradiation at 365 nm was diluted to 20 μL with reaction buffer and transferred to a 1 cm^2 chambered microscope slide (Lab-Tek, Miles Inc.). The solution was irradiated at room temperature for 20 min with a germicidal lamp. The solution was then evaporated and the residue subjected to polyacrylamide gel electrophoresis. Wet gels were autoradiographed at −80 °C. The autoradiograms were scanned with an LKB Ultrascan XL laser densitometer to quantitate the extent of photo-cross-linking.

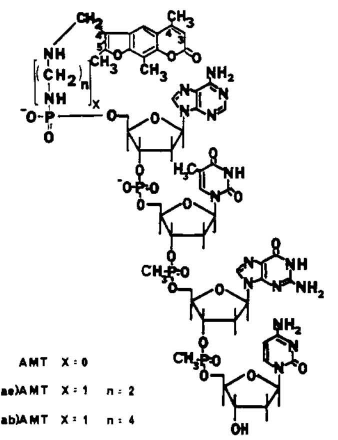

FIGURE 1: General structure of deoxyribonucleoside methylphosphonates derivatized with 4′-(aminoalkyl)-4,5′,8-trimethylpsoralen.

$^{3'}\mathrm{D\text{-}CCGGAGACGTATTTATTTTTTTTAATCAGTCGGTA_p{}^{32}}{}^{5'}$	**1**
$\mathrm{A_p\underline{ATAAAAAAAAT}}$	I
$\mathrm{AMT_pA_p\underline{ATAAAAAAAAT}}$	AMT-I
$\mathrm{(ae)AMT_pA_p\underline{ATAAAAAAAAT}}$	(ae)AMT-I
$\mathrm{(ab)AMT_pA_p\underline{ATAAAAAAAAT}}$	(ab)AMT-I
$\mathrm{(ae)AMT_pA_p\underline{ATAACAAAAAT}}$	(ae)AMT-III
$\mathrm{(ae)AMT_pC_p\underline{ATAAAAAAAAT}}$	(ae)AMT-IV
$\mathrm{(ae)AMT_pA_p\underline{GTGAGAGAGAT}}$	(ae)AMT-V
$\mathrm{(ae)AMT_pA_p\underline{ATAAAACTCTC}}$	(ae)AMT-VI
$\mathrm{(ae)AMT_pA_p\underline{ATAACTCTCTC}}$	(ae)AMT-VII
$^{3'}\mathrm{D\text{-}TACCGACTGATTAAAAAAAATAAATACGTCTCCGG_p{}^{32}}{}^{5'}$	**2**
$\mathrm{A_p\underline{TTTTTTTTATTTAT}}$	II
$\mathrm{AMT_pA_p\underline{TTTTTTTTATTTAT}}$	AMT-II
$\mathrm{(ae)AMT_pA_p\underline{TTTTTTTTATTTAT}}$	(ae)AMT-II
$\mathrm{(ab)AMT_pA_p\underline{TTTTTTTTATTTAT}}$	(ab)AMT-II

FIGURE 2: Nucleotide sequences of single-stranded DNA 35-mers and their complementary 4′-(aminoalkyl)-4,5′,8-trimethylpsoralen-derivatized methylphosphonate oligomers. The designation for each oligomer is given at the right side of the oligomer. The underline indicates the position of the methylphosphonate linkages.

RESULTS

Synthesis and Characterization of 4′-(Aminoalkyl)-4,5′,8-trimethylpsoralen-Derivatized Oligodeoxyribonucleoside Methylphosphonates. The general structure of the (aminoalkyl)-4,5′,8-trimethylpsoralen-derivatized methylphosphonate oligomers is shown in Figure 1. It should be noted that the first internucleotide bond at the 5′ end of the oligomer is a phosphodiester linkage. The methylphosphonate oligomers are derivatized with either 4′-(aminomethyl)-4,5′,8-trimethylpsoralen (AMT), 4′-[[N-(2-aminoethyl)amino]methyl]-4,5′,8-trimethylpsoralen [(ae)AMT], or 4′-[[N-(4-aminobutyl)amino]methyl]-4,5′,8-trimethylpsoralen [(ab)AMT] through a 5′-phosphoramidate linkage. As shown in Figure 2, the oligomers are complementary to two oligodeoxyribonucleotide 35-mers whose nucleotide sequences correspond to nucleotides 6–40 in the origin of replication region of SV40 DNA. In addition to these completely com-

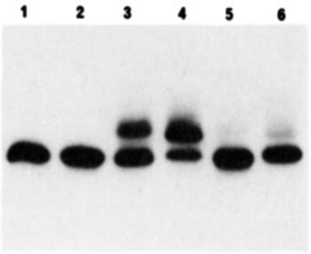

FIGURE 3: Photoadduct formation between AMT-derivatized methylphosphonate oligomers and single-stranded DNA 35-mers. A solution containing 10 μM oligomer and 0.15 μM 35-mer in 10 mM Tris at pH 7.5 was irradiated at 365 nm for 20 min at 4 °C and in the case of lanes 5 and 6 at 254 nm for another 20 min at 22 °C. The reaction mixtures were subjected to polyacrylamide gel electrophoresis. The autoradiogram shows the following reactions: lane 1, **2** and AMT-II; lane 2, **1** and AMT-I; lane 3, AMT-II and **2** irradiated at 365 nm; lane 4, AMT-I and **1** irradiated at 365 nm; lane 5, AMT-II and **2** irradiated at 365 and 254 nm; lane 6, AMT-I and **1** irradiated at 365 and 254 nm.

plementary methylphosphonate oligomers, a series of oligomers were synthesized which contained noncomplementary bases at specific positions in the oligomers. The base sequences of the 35-mers and the binding sites of the corresponding methylphosphonate oligomers are shown in Figure 2. For ease of reference, the 35-mers will be designated by an Arabic number **1** or **2**. The sequences of the methylphosphonate oligomers will be designated by a Roman numeral prefixed with the symbol of the psoralen derivative.

The psoralen-derivatized methylphosphonate oligomers were prepared by reaction of 5′-phosphorylated oligonucleoside methylphosphonates with 4′-(aminoalkyl)-4,5′,8-trimethylpsoralen in the presence of a water-soluble carbodiimide in 2,6-lutidine hydrochloride buffer. The conversion of the phosphorylated oligomer to the psoralen-derivatized oligomer appeared to be complete after 2 days at room temperature. The oligomers were purified by either gel electrophoresis or DEAE-cellulose chromatography. The mobility of the psoralen-derivatized oligomer on polyacrylamide gels was less than that of the 5′-phosphorylated oligomer due to the reduced negative charge and increased molecular weight of the molecule.

To further characterize the psoralen-derivatized oligomers, d-AMT–[^{32}P]pTp*GCACCAT* was synthesized. Treatment of this oligomer with 0.1 M hydrochloric acid at 37 °C for 4 h resulted in formation of d-[^{32}P]pTp*GCACCAT* as shown by polyacrylamide electrophoresis. Prolonged incubation of d-AMT–[^{32}P]pTp*GCACCAT* with S_1 nuclease resulted in the formation of a new compound whose mobility on polyacrylamide gel electrophoresis, paper chromatography, silica gel TLC, or reversed-phase TLC was identical with that of d-AMTpT. The latter compound was synthesized by reaction of AMT with d-pT and was characterized by ultraviolet spectroscopy and proton NMR spectroscopy.

Photoadduct Formation between 4′-(Aminoalkyl)-4,5′,8-trimethylpsoralen-Derivatized Oligonucleoside Methylphosphonates and Oligodeoxyribonucleotides **1** *and* **2**. The photoreactions between 35-mers **1** and **2** and their complementary AMT-derivatized methylphosphonate oligomers were studied at 4 °C. Irradiation of a solution of AMT-I and 35-mer **1** or of AMT-II and 35-mer **2** in 10 mM Tris buffer at 365 nm resulted in formation of a new ^{32}P-labeled compound as shown by polyacrylamide gel electrophoresis (Figure 3). Irradiation of **1** or **2** alone had no effect on the mobility of the 35-mer (data not shown). When the reaction solutions were further irradiated at 254 nm, the new photoadducts disappeared, and the original 35-mers were regenerated as shown in lanes 5 and 6 of Figure 3. Similar photoadduct formation was observed when **1** and (ae)AMT-I or (ab)AMT-I or when **2** and (ae)AMT-II or (ab)AMT-II were irradiated. No photoadduct formation was observed when (ae)AMT-I and **2** or when (ae)AMT-II and **1** were irradiated.

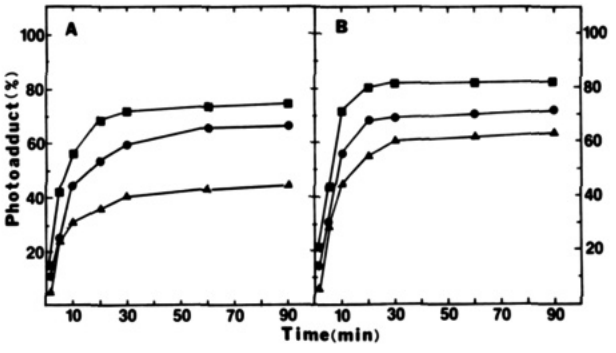

FIGURE 4: Kinetics of photoadduct formation between psoralen-derivatized methylphosphonate oligomers and single-stranded DNA 35-mers. The reaction conditions were the same as described in Figure 3. Panel A shows reactions between AMT-II and **2** (▲), (ae)AMT-II and **2** (■), and (ab)AMT-II and **2** (●). Panel B shows reactions between AMT-I and **1** (▲), (ae)AMT-I and **1** (■), and (ab)AMT-I and **1** (●).

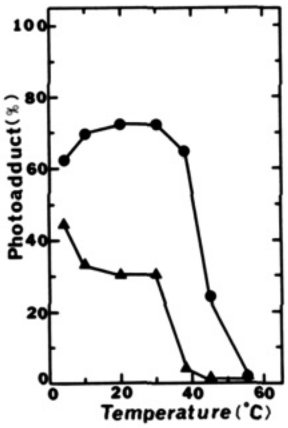

FIGURE 5: Effect of temperature on the cross-linking reaction between AMT-I and **1** (●) or AMT-II and **2** (▲). The reaction conditions were the same as those described in Figure 3.

The kinetics of cross-linking between 35-mers **1** or **2** and their complementary psoralen-derivatized methylphosphonate oligomers were determined and are shown in Figure 4. The extent of the cross-linking reaction appeared to level off after 20-min irradiation for each 35-mer/methylphosphonate oligomer combination. The rate and extent of photo-cross-linking for both methylphosphonate oligomers followed the order (ae)AMT > (ab)AMT > AMT. When AMT-I or AMT-II was preirradiated for 20 min at 4 °C prior to addition to **1** or **2**, respectively, and the AMT oligomer/35-mer mixture was then irradiated for 20 min, 20% adduct formation was observed for AMT-I and no adduct formation was observed for AMT-II.

The concentrations of the psoralen-derivatized oligomers were 10 μM in the experiments described above. With all three types of psoralen derivatives, the extent of cross-linking after 20 min of irradiation at 4 °C increases over the concentration range 1–5 μM. Above 5 μM, however, the extent of cross-linking remains essentially constant.

The above experiments were all carried out at 4 °C. Cross-linking experiments with 10 μM AMT-I or AMT-II and 0.15 μM 35-mer were carried out over the temperature range 4–55 °C. The results of these experiments are shown in Figure 5. The extent of cross-linking by AMT-I was greater than

Table I: Extent of Cross-Linking between 35-mer **1** and (ae)AMT-Derivatized Oligonucleoside Methylphosphonates[a]

oligomer	% cross-linked 35-mer	oligomer	% cross-linked 35-mer
(ae)AMT-I	85	(ae)AMT-V	33
(ae)AMT-III	53	(ae)AMT-VI	19
(ae)AMT-IV	58	(ae)AMT-VII	0

[a] The reactions were carried out under the conditions described in Figure 3.

that of AMT-II over the entire temperature range studied. The percentage of photoadduct formation by AMT-I remained approximately constant at 70% up to 40 °C and then rapidly decreased to 0% by 55 °C. In contrast, the extent of cross-linking by AMT-II remained fairly constant at 30% over the temperature range 10–30 °C and then dropped off to 0% above this temperature.

In a separate series of experiments, the melting temperatures of the 1:1 duplexes formed between underivatized methylphosphonate oligomers and their complementary 35-mers were measured in 10 mM Tris buffer at a total strand concentration of 2 μM. The duplex formed between **1** and **2** melted at 48 °C. The melting temperature of the duplex formed by I and **1** was 29 °C whereas that of the duplex formed by II and **2** was 16 °C. No increase in T_m was observed for the psoralen-derivatized methylphosphonate oligomers and their complementary 35-mers.

The effect of noncomplementary bases on the cross-linking reaction between (ae)AMT-derivatized methylphosphonate oligomers and 35-mer **1** is shown in Table I. The sequences of these oligomers each include part of the original sequence of the parent oligomer, (ae)AMT-I. Cross-linking is reduced approximately 30% for (ae)AMT-III and (ae)AMT-IV which contain a single noncomplementary nucleoside in the center or the 5′ end of the oligomer, respectively. Oligomer (ae)-AMT-V will form 5 GT base pairs with **1**, and the extent of its cross-linking is reduced 50% relative to (ae)AMT-I. Substituting the five nucleosides at the 3′ end of (ae)AMT-I with C and T residues to give (ae)AMT-VI reduces the cross-linking to 19% whereas no cross-linking occurs with (ae)AMT-VII in which the number of noncomplementary nucleosides at the 3′ end is increased to seven. The completely noncomplementary (ae)AMT-II does not cross-link with **1**. In a separate experiment, we showed that d-AMTpTp*GTTGGTCTTGT* which is partially complementary to **2** does not cross-link with **2**. In the cases where partially complementary oligomers did cross-link, we demonstrated that adduct formation could be completely reversed by additional irradiation at 254 nm.

DISCUSSION

Derivatives of psoralen have been used to study nucleic acid secondary structure (Song & Tapley, 1979; Cimino et al., 1985). Psoralens interact via intercalation with double-stranded regions of DNA or RNA. The interaction of DNA and RNA with 4′-(aminomethyl)-4,5′,8-trimethylpsoralen (AMT) has been studied extensively by Hearst and co-workers (Isaacs et al., 1977; Hearst, 1981). Upon irradiation with long-wavelength UV light, a cycloaddition reaction occurs between the 5,6 double bond of pyrimidine residues and either the 4′,5′ double bond of the psoralen furan ring or the 3,4 double bond of the psoralen pyrone ring to give a psoralen–nucleic acid monoadduct. Under appropriate conditions, the furan side monoadduct can undergo another cycloaddition with a pyrimidine of the complementary nucleic acid strand, resulting in psoralen-cross-linked nucleic acid. The structures of these photoadducts have been determined (Straus et al., 1981; Kanne et al., 1982; Peckler et al., 1982).

Recently, Hearst and co-workers have prepared oligonucleotides containing furan side psoralen monoadducts (van Houten et al., 1986; Shi & Hearst, 1987a). The position of the psoralen residue is generally in the middle of the oligomer sequence. Upon irradiation, these oligonucleotides cross-link with complementary sequences of a nucleic acid.

We have prepared a series of oligonucleoside methylphosphonates in which 4′-(aminoalkyl)-4,5′,8-trimethylpsoralens are linked to the 5′ end of the oligomers via phosphoramidate bonds. The distance between the trimethylpsoralen ring and the end of the oligomer can be adjusted by means of the aminoalkyl linker arm (Figure 1). The ability of the psoralen-derivatized methylphosphonate oligomers to cross-link to target single-stranded DNAs was studied. The target DNAs are 35 nucleotides in length, and their sequences correspond to both strands of the AT-rich region of the origin of replication of SV40 DNA. The sequences of the methylphosphonate oligomers were designed such that the 5′-psoralen group of the oligomer opposes a thymidine residue in the target 35-mer (Figure 2). Examination of molecular models shows the psoralen ring of the methylphosphonate oligomer can stack upon the opposing thymine ring when the oligomer is correctly hydrogen bonded to the 35-mer. The 3,4 double bond of the psoralen pyrone ring appears to be sufficiently close to the thymine 5,6 double bond for cycloaddition to occur when the duplex is irradiated.

Phosphorylated methylphosphonate oligomers were prepared enzymatically by using polynucleotide kinase. These phosphorylation reactions have been carried out on scales up to 90 nmol and can be used to prepare nonradioactive as well as ^{32}P-labeled oligomers. The psoralen-derivatized oligomers were prepared by reaction of the phosphorylated oligomers with 4′-(aminoalkyl)-4,5′,8-trimethylpsoralen in the presence of a water-soluble carbodiimide. The conditions for this reaction are similar to those described by Chu et al. (1983) for the coupling of primary amines to 5′-phosphorylated oligodeoxyribonucleotides. However, we found that the reaction proceeds satisfactorily in lutidine buffer alone and did not require the formation of an imidazolide intermediate.

The cross-linking reaction was monitored by polyacrylamide gel electrophoresis under denaturing conditions. Upon irradiation of a solution of ^{32}P-labeled 35-mer and its complementary psoralen-derivatized methylphosphonate oligomer by 365-nm UV light, a new band appeared on the gel whose mobility was less than that of the original 35-mer (Figure 3). This new band is the photoadduct formed by the 35-mer and the methylphosphonate oligomer. The decreased mobility of the new band reflects the increased molecular weieght of the 35-mer/methylphosphonate oligomer photoadduct. The mobility of the 35-mer did not change when it was irradiated in the absence of the psoralen-derivatized methylphosphonate oligomer or if it was irradiated in the presence of a noncomplementary psoralen-derivatized oligomer. This behavior shows that 365-nm light does not induce cross-linking between 35-mer strands and that photoadduct formation by the psoralen-derivatized methylphosphonate oligomer is strictly dependent upon its ability to hydrogen bond to the target nucleic acid.

Formation of pyrimidine/psoralen photoadducts can be reversed by irradiation at 254 nm (Shi & Hearst, 1987b). When the 35-mer/methylphosphonate oligomer photoadduct was irradiated at 254 nm, the photoadduct disappeared, and the original 35-mer was regenerated (Figure 3). This behavior is consistent with the formation of a cyclobutane bridge between the thymine ring of the 35-mer and the psoralen ring of the methylphosphonate oligomer. Examination of molecular models suggests that the photoadduct is most likely formed by the pyrone ring of the psoralen and that it is in the cis-syn configuration. This is the same configuration observed for thymidine/psoralen photoadducts isolated after irradiation of DNA and psoralens (Straub et al., 1981; Kanne et al., 1982; Peckler et al., 1982). Formation of adducts by the furan ring of the psoralen or formation of adducts of other configurations does not appear possible because of the steric constraints imposed on the system by the attachment of the psoralen group to the 5′ end of the methylphosphonate oligomer.

The length of the aminoalkyl linker arm affects both the rate and the extent of cross-linking of the methylphosphonate oligomers to the 35-mer (Figure 4). Molecular models suggest that the aminomethyl linker of the psoralen-derivatized oligomers is the minimal length which will allow interaction between the 35-mer thymine and the psoralen pyrone ring when the 35-mer/methylphosphonate oligomer duplex is in a B form. The models predict that the *N*-(2-aminoethyl)-aminomethyl and the *N*-(4-aminobutyl)aminomethyl linkers should allow the trimethylpsoralen ring to extend further toward the thymine ring and thus should allow for greater overlap between the pyrone and the thymine ring. The observed extents of cross-linking, which follow the order (ae)-AMT > (ab)AMT > AMT, are consistent with these predictions. The decreased cross-linking of the (ab)AMT-derivatized oligomers relative to the (ae)AMT-derivatized oligomers probably reflects the greater number of degrees of freedom of the psoralen ring in the (ab)AMT oligomers.

The extent of cross-linking increased during the first 20 min of irradiation and then leveled off (Figure 4). Although the extent of cross-linking after 20 min increased with increasing oligomer concentration over the range 1–5 μM, further increases in the oligomer concentration did not further increase the amount of cross-linked product. Thus quantitative cross-linking was not observed even in the presence of a 50–200-fold excess of the psoralen oligomer. This behavior suggests that the psoralen-derivatized oligomers are inactivated during the irradiation. Consistent with this explanation is the observation that preirradiation of AMT-I or AMT-II at 365 nm prior to the cross-linking experiment reduces or prevents cross-linking.

Previous studies on AMT have shown that it is converted to a cross-linking-inactive product when irradiated at 365 nm (Hearst, 1981). AMT photodeactivation and photo-cross-linking with DNA proceed with similar quantum efficiences. Thus, in our reactions, it appears that the psoralen-derivatized oligomers undergo two photoreactions: cross-linking with the 35-mer and photodeactivation. Under conditions of high oligomer concentration, it is likely that the 35-mer is saturated with psoralen-derivatized methylphosphonate oligomer. Thus, it appears that the rate of photodeactivation of the oligomer in either the free or the bound form is the same as or greater than its overall rate of binding and photo-cross-linking to the 35-mer.

As shown in Figure 5, the observed effect of temperature on the cross-linking reactions is qualitatively similar to the melting behavior of the duplexes formed between the methylphosphonate oligomers and the 35-mers. The melting temperature of the duplex formed by 35-mer **1** and oligomer I, which is 12 nucleotides long, is 13 °C higher than that formed by 35-mer **2** and oligomer II, which is 15 nucleotides in length. We have previously observed that oligo(dT) methylphosphonates form rather low-melting duplexes with poly(dA) (Miller et al., 1981). It appears that the T_m of duplexes formed by oligo(dT) methylphosphonates may be particularly sensitive to the configuration of the methylphosphonate linkage (Miller et al., 1980). This may explain why duplexes formed by II, which consists of 12 thymidine residues, have T_m's lower than do those of I, which consists of predominantly A residues.

The (ae)AMT oligomers form duplexes which have the same T_m's as those of the underivatized methylphosphonate oligomers. The measured binding constant for the interaction of 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen with DNA is approximately 3×10^4 M^{-1} (Isaacs et al., 1977). This binding constant is approximately 1–2 orders of magnitude less than that of the binding constants predicted for oligomers I or II with a complementary nucleic acid. Thus, it appears that the psoralen group does not contribute significantly to the binding of the oligomers to the 35-mers. This behavior is in contrast to the effects of adding acridine to the 3′ end of oligodeoxyribonucleotides, which results in a significant increase in the T_m of duplexes formed by these oligomers (Asseline et al., 1984; Lancelot et al., 1985).

Although the stability of duplex formation is not affected by the presence of the psoralen group, the extent of cross-linking is strongly dependent upon the fidelity of the hydrogen-bonding interactions between the psoralen-derivatized methylphosphonate oligomer and the 35-mer. This was demonstrated by preparing oligomers which contain noncomplementary bases (Table I). Inclusion of a single noncomplementary base either in the middle or at the 5′ end of the oligomer decreased the extent of cross-linking by approximately 30% after 20 min of irradiation even at 4 °C. These results suggest that a single mismatch significantly destabilizes duplex formation and are consistent with previous results which showed that the biological and biochemical activities of mismatched methylphosphonate oligomers are considerably reduced relative to the perfectly matched oligomer (Smith et al., 1986).

A further reduction in cross-linking was observed when the number of mismatches was increased or when GT base formation was allowed. In the case of (ae)AMT-VII, the psoralen-derivatized 5′ end of the oligomer could potentially form a partial duplex containing five base pairs. However, it appears that if partial binding did occur it was not sufficiently stable to allow cross-linking to proceed even at 4 °C. Similarly, no cross-linking was observed when a solution of 35-mer **2** and d-AMTpTp*GTTGGTCTTGT*, which could form six random AT base pairs, was irradiated.

Oligonucleotides capable of cross-linking with complementary nucleic acid sequences are potentially useful probes for studying and controlling gene expression at the RNA or possibly DNA level. Oligodeoxyribonucleotides derivatized with various alkylating groups have been prepared, and the ability of these oligomers to cross-link to complementary nucleic acids has been extensively studied (Knorre & Vlassov, 1985). Although oligomers of this type cross-link with a reasonable degree of efficiency, the oligomers have been observed to bind to multiple sites on targeted nucleic acids (Gorshkova et al., 1986). This may be due to the presence of the highly reactive alkylating groups which can cross-link even when the oligomer is transiently bound to a partially complementary site. Psoralen-derivatized oligomers can cross-link only when activated with long-wavelength UV light and only when the psoralen is properly positioned with respect to a pyrimidine base in the target strand of the oligomer. The binding interaction is governed solely by the hydrogen-bonding properties of the oligomer sequence. Thus, the psoralen-derivatized oligomers offer potentially greater specificity in cross-linking reactions than do oligomers derivatized with alkylating agents.

The results of the experiments described here demonstrate that quite high extents of cross-linking are achievable with (ae)AMT-derivatized methylphosphonate oligomers. Preliminary results (Murakami et al., 1986) to be described in more detail in a future publication show that psoralen-derivatized methylphosphonate oligomers specifically cross-link to mRNA and as result inhibit mRNA translation in cell-free systems. These properties combined with the known ability of methylphosphonate oligomers to be taken up by living cells suggest that psoralen-derivatized oligonucleoside methylphosphonates may be useful probes of cellular gene expression.

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