Effect of Target Structure on Cross-Linking by Psoralen-Derivatized Oligonucleoside Methylphosphonates†

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Received February 25, 1994; Revised Manuscript Received May 24, 1994®

ABSTRACT: A series of psoralen-derivatized oligodeoxyribonucleoside methylphosphonates were examined for their abilities to cross-link to DNA and RNA oligonucleotide targets. These targets were designed to have either a random coil or a hairpin structure in solution. The methylphosphonate oligomers cross-linked with approximately the same rates to the random coil DNA and RNA targets, although the extent of cross-linking to the DNA target was higher than that to the RNA target. For a given methylphosphonate sequence, cross-linking decreased as the temperature increased, and this behavior paralleled the interaction of the oligomer with the target as determined by ultraviolet melting experiments. The oligomers also cross-linked efficiently with the DNA hairpin target, but little or no cross-linking was observed with the RNA hairpin. In the case of these hairpin targets, the extent of cross-linking was dependent upon the location of the oligomer binding site relative to the stem and loop regions of the hairpin. The lack of reactivity with the RNA hairpin may be due to the high stability of the stem of this target versus that in the DNA target and the relatively lower efficiency of binding of the methylphosphonates to RNA versus DNA targets. The sequences of the oligomers are complementary to vesicular stomatitis virus M-protein mRNA. One of the oligomers was tested, and was found to cross-link at 20 °C to VSV N-mRNA to approximately the same extent as observed for cross-linking with the random coil RNA target, suggesting that the mRNA binding site for the oligomer most likely is in a somewhat open conformation. The results of our experiments suggest that target structure and sequence can have a significant influence on binding/cross-linking reactions of psoralen-derivatized oligonucleoside methylphosphonates and that these parameters will most likely influence binding/cross-linking of these oligomers to cellular RNA targets such as mRNA.

Conjugation of antisense or antigene oligodeoxyribonucleotides or oligonucleoside methylphosphonates with derivatives of the photoreactive cross-linking functional group psoralen provides an effective means to covalently attach the oligomer to complementary nucleic acid targets. A number of studies have shown that such psoralen-derivatized oligomers can efficiently cross-link *in vitro* with single-stranded DNA (Gamper *et. al.*, 1984, 1987; Houten *et al.*, 1986b,b; Shi & Hearst, 1987; Cheng *et al.*, 1988; Lee *et al.*, 1988a,b; Pieles & English, 1989; Bhan & Miller, 1990; Woo & Hopkins, 1991; Reynolds *et al.*, 1992; Kean & Miller, 1993), with RNA (Kean *et al.*, 1988; Teare & Wollenzien, 1989, 1990), and with double-stranded DNA (Takasugi *et al.*, 1991; Duval-Valentin *et al.*, 1992; Giovannangeli *et al.*, 1992). These cross-linking reactions are of potential utility for enhancing the effectiveness of antisense oligonucleotides (Miller, 1992a).

Psoralen-derivatized oligonucleoside methylphosphonates can cross-link to cellular and viral mRNAs with high degree of sequence specificity (Kean *et al.*, 1988; Levis, 1993). Although the specificity of cross-linking can be controlled by proper choice of oligomer sequence, the extent to which a particular oligomer cross-links is less predictable. This is most likely due to the presence of secondary and tertiary structure within the mRNA. Such structure may limit access of antisense oligomers to their complementary binding sites or affect the ability of the oligomer to completely interact with its binding site (Chastain & Tinoco, 1993; Freier, 1993). Little is known about the secondary structure of naturally occurring RNAs, and there is only limited experimental information on the effects of RNA secondary structure on oligonucleotide binding interactions and/or antisense activities (Gamper *et al.*, 1987; Bacon & Wickstrom, 1991; Ecker *et al.*, 1992; Hjalt *et al.*, 1992; Lima *et al.*, 1992; Stull *et al.*, 1992; Rittner *et al.*, 1993). It appears that binding of oligonucleotides containing phosphodiester or phosphorothioate internucleotide linkages to RNA hairpins is strongly influenced by the location of the oligomer binding site in the target RNA and by the nature of the oligomer backbone (Ecker *et al.*, 1992; Lima *et al.*, 1992). It seems likely that target structure should also influence the binding/cross-linking reactions of psoralen-derivatized oligonucleoside methylphosphonates. To explore this possibility, we have examined *in vitro* cross-linking reactions between psoralen-derivatized oligonucleoside methylphosphonates and simple DNA and RNA oligonucleotide target molecules.

EXPERIMENTAL PROCEDURES

[γ-^{32}P]ATP was purchased from Amersham Inc. T4 polynucleotide kinase, RNase T_1, RNase CL_3, and S1 nuclease were purchased from United States Biochemical Corp. Protected deoxyribonucleotide and ribonucleoside phosphoramidites were purchased respectively from Cruachem and MilliGen/Biosearch. Protected deoxyribonucleotide phosphonamidites were a gift from JBL Scientific, San Luis Obispo, CA. Some of the r-I RNA used in these experiments was a gift from Genta Inc. Sep-Pak C-18 reversed-phase cartridges were obtained from Waters Associates. Polyacrylamide gel electrophoresis was carried out on 20% polyacrylamide gels containing 7 M urea. The gels were run in TBE buffer which contained 0.089 M tris(hydroxymethyl)aminomethane (Tris),[1] 0.089 M boric acid, and 0.2 mM EDTA buffered at pH 8.0. Unless otherwise stated, gel loading buffer contained 90%

† Supported by a grant from the National Cancer Institute (CA 42762).
® Abstract published in *Advance ACS Abstracts,* July 15, 1994.

0006-2960/94/0433-9178$04.50/0

formamide, TBE, 0.05% xylene cyanol, and 0.05% bromophenol blue. Wet gels were autoradiographed at −80 °C. All chemicals used were reagent grade or better. All 5′-^{32}P end-labeled oligomers were gel-purified.

Syntheses of Oligodeoxyribonucleotides, Oligoribonucleotides, and Oligodeoxyribonucleoside Methylphosphonates. The oligodeoxyribonucleotides, oligoribonucleotides, and oligodeoxyribonucleoside methylphosphonates were prepared in a Biosearch Model 8700 DNA synthesizer on 1 μmol scales on controlled pore glass supports using base-protected monomers. The synthesizer was programmed to remove the 5′-terminal dimethoxytrityl group from the protected oligomers at the end of the synthesis. Oligodeoxyribonucleotides were deprotected and removed from the support following literature procedures (Brown & Brown, 1991). Deprotection of oligodeoxyribnonucleoside methylphosphonates was carried out according to previously published procedures (Miller *et al.*, 1991), or in some cases the oligomers were deprotected according to Hogrefe *et al.* (1993). Oligoribonucleotides were deprotected and removed from the support using a modification of the procedure of Scaringe *et al.* (1990). The support was treated with 1 mL of ethanolic ammonia for 18 h at 55 °C, removed by filtration, and washed 3 times with 0.5 mL of ethanol/water (1:1 v/v). The solvents were combined and evaporated, and the residue was treated with 0.6 mL of 1 M tetrabutylammonium fluoride in tetrahydrofuran (Aldrich) for 48 h at 20 °C. The reaction mixture was diluted with 10 mL of 0.1 M triethylammonium bicarbonate, pH 7.0, and loaded onto a Qiagen anion-exchange cartridge (Qiagen Inc.) that had been equilibrated with 10 mL of 0.1 M triethylammonium bicarbonate. After the cartridge was washed with 5 mL of 0.1 M triethylammonium bicarbonate, the oligomer was eluted with 7 mL of 2 M triethylammonium bicarbonate and desalted on C-18 Sep-Pak cartridges which had been equilibrated with 10 mL of acetonitrile, 10 mL of acetonitrile/water (1:1 v/v), and 10 mL of 0.1 M sodium phosphate, pH 5.8. The Sep-Pak was washed with 10 mL of water, and the oligomer eluted with 3 mL of acetonitrile/water (1:1 v/v). In order to determine whether deprotection was complete, oligoribonucleotides were end-labeled using [γ-^{32}P]ATP and polynucleotide kinase and analyzed on 30 cm × 40 cm × 0.4 cm polyacrylamide gels. Electrophoresis of r-I and r-II was carried out at 1800 V for 5.5 and 6.5 h, respectively, so that full-length oligomer had migrated at least two-thirds the length of the gel. In our experience, oligomer that migrates as one band on a 14 cm × 16 cm × 0.75 cm gel may actually appear as a collection of closely spaced bands on the longer gel. We attribute these slight differences in mobility to different degrees of deprotection of the full-length oligoribonucleotide.

Psoralen Derivatization of Oligodeoxyribonucleoside Methylphosphonates. Psoralen-derivatized oligodeoxyribonucleoside methylphosphonates were prepared according to literature procedures (Bhan & Miller, 1990; Miller, 1992b). Oligomers were phosphorylated using ATP and polynucleotide kinase, and then converted to the imidazole derivative by treatment with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (Sigma Chemicals) in 0.1 M imidazole buffer at pH 6.0. The imidazolide was reacted with 4′-[[*N*-(2-aminoethyl)-amino]methyl]-4,5′,8-trimethylpsoralen for 48 h at 37 °C in 0.25 M lutidine hydrochloride buffer at pH 7.5, and the psoralen-derivatized product was purified by preparative C-18 reversed-phase chromatography on a 1.0 cm × 25 cm Rainin Microsorb column.

[1] Abbreviations: VSV, vesicular stomatitis virus: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate.

Melting Experiments. The melting experiments were carried out in 0.1 M sodium chloride/50 mM Tris, pH 7.6. Hairpins were formed by heating 1.0 mL solutions of d-II and r-II for 5 min at 65 °C, cooling to 20 °C for 20 min, and then cooling for 15 min on ice. Duplexes were formed by mixing 0.5 mL of equimolar solutions of the oligomers and incubating overnight at 4 °C. The melting transitions were measured either using a Varian 219 UV/vis spectrophotometer fitted with a thermostated cell holder connected to a Neslab RTE 100 circulating, programmable temperature bath or on a Cary 3E UV/vis spectrophotometer fitted with a thermostated cell block and temperature controller. At low temperature, the cell compartment was continuously purged with dry nitrogen to prevent condensation. The solutions were heated at a rate of 0.5 °C/min, and the A_{260} was recorded as a function of temperature.

Potassium Permanganate Reactions. Ten microliter solutions containing either 0.1 μM 5′-^{32}P end-labeled target DNA [(3–4) × 10^4 dpm] or 0.1 μM 5′-^{32}P end-labeled target DNA and 5 μM psoralen-derivatized oligodeoxyribonucleoside methylphosphonate in 0.1 M sodium chloride/50 mM Tris, pH 7.6, were treated with 70 μM potassium permanganate for 30 min at 20 °C, after which the solution was treated with 2.5 μL of allyl alcohol, and then treated with 40 μL of 1.25 M piperidine for 30 min at 90 °C (Kean & Miller, 1993). The piperidine was lyophilized, and the residue was lyophilized twice from 10 μL of water and dissolved in 6 μL of gel loading dye. The reactions were then analyzed by gel electrophoresis on a 30 cm × 40 cm × 0.4 cm polyacrylamide gel for 2 h at 1800 V. Before reaction of solutions containing d-II, the hairpin molecule was renatured in 0.1 M sodium chloride/50 mM Tris, pH 7.6, as described above.

Nuclease Digestion Experiments. Approximately 40 000 dpm of either 5′-^{32}P end-labeled d-I or 5′-^{32}P end-labeled d-II was renatured as described above in 4 μL of a buffer containing 0.1 M sodium chloride, 50 mM Tris, 1 mM zinc chloride, pH 7.5, and 2 μg of carrier tRNA. After the addition of 0.5–10 units of S1 nuclease, the solution was incubated for 10 min at 4 °C. The reactions were stopped by the addition of 0.5 μL of 0.1 M EDTA, lyophilized, dissolved in 10 μL of gel loading dye, and subjected to gel electrophoresis on a 30 cm × 40 cm × 0.4 cm gel for 2 h at 1800 V.

After renaturation in 0.1 M sodium chloride/50 mM Tris, pH 7.6, as described above, 0.1 μM 5′-^{32}P end-labeled r-I or r-II (approximately 40 000 dpm) and 0–100 μM psoralen-derivatized oligodeoxyribonucleoside methylphosphonate were digested with 0.04–1.0 unit of RNase T_1 or RNase CL_3 for 30 min at 4 °C in 5 μL of a solution containing 0.1 M sodium chloride, 50 mM Tris, pH 7.6, and 1 μg of carrier tRNA. The reaction was stopped by incubation in a dry ice bath, diluted with 5 μL of gel loading buffer containing 9.8 M urea, 1.5 mM EDTA, and 0.05% xylene cyanol, and subjected to gel electrophoresis on a 30 cm × 40 cm × 0.4 cm gel for 2 h at 1800 V. An RNase T_1 digest in 6 M urea and an alkaline hydrolysate were run for reference.

Polyacrylamide Gel Shift Mobility Assays. Solutions containing the following 5′-^{32}P end-labeled oligodeoxyribonucleotides were renatured in 5 μL of 0.1 M sodium chloride/50 mM Tris, pH 7.6, as described above: 0.1 μM d-II, 0.1 μM d-III, 0.1 μM d-IV, or 0.1 μM d-III and 0.1 μM d-IV. The solutions were diluted with an equal volume of ice-cold loading buffer containing 50% glycerol (v/v), 10 mM Tris, pH 7.6, 0.2% xylene cyanol, and 0.2% bromophenol blue and subjected to gel electrophoresis at 4 °C on a 20% nondenaturing 14 cm × 16 cm × 0.75 cm polyacrylamide gel for 6 h at 250 V.

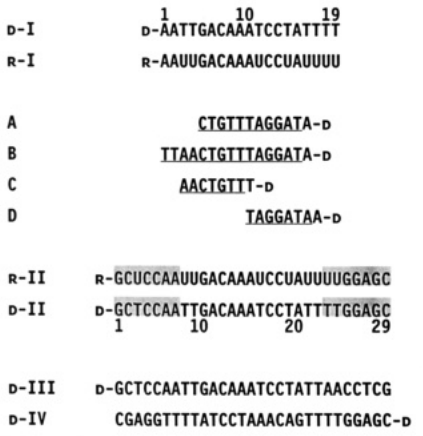

FIGURE 1: Schematic of linear and hairpin oligonucleotide targets and their complementary oligonucleoside methylphosphonates. The underline indicates the positions of the methylphosphonate linkages.

Identical samples were also gel electrophoresed on 20% polyacrylamide gels containing 7 M urea for 2 h at 800 V.

Cross-Linking Experiments with Psoralen-Derivatized Oligodeoxyribonucleoside Methylphosphonates. Solutions containing 0.1 μM 5′-^{32}P end-labeled oligo RNA or DNA and 0–100 μM psoralen-derivatized oligodeoxyribonucleoside methylphosphonate in 10 μL of 0.1 M sodium chloride/50 mM Tris, pH 7.6, were preincubated in borosilicate glass test tubes (Corning) at the temperature of irradiation. Each tube was then irradiated at 365 nm for 0–60 min at 4–55 °C 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 does not transmit light below 300 nm. The irradiated mixtures were lyophilized, dissolved in 10 μL of gel loading buffer, and subjected to gel electrophoresis on a 14 cm × 16 cm × 0.75 cm gel for 2 h at 800 V. For irradiations with d-II or with r-II, the hairpin molecule was first preformed by renaturing as described above at 0.2 μM either in a solution containing 0.1 M sodium chloride/50 mM Tris, pH 7.6, and then 5 μL of this solution was combined with 5 μL of a solution of 10 μM psoralen-derivatized oligodeoxyribonucleoside methylphosphonate in 0.1 M sodium chloride/50 mM Tris, pH 7.6, or in a solution containing both 0.1 μM target and 5 μM psoralen-derivatized oligodeoxyribonucleoside methylphosphonate in 0.1 M sodium chloride/50 mM Tris, pH 7.6. In either case, no differences in cross-linking were observed.

RESULTS

Oligonucleotide Targets and Their Complementary Oligonucleoside Methylphosphonates. The sequences of the oligodeoxyribonucleotide and oligoribonucleotide targets and their complementary oligodeoxyribonucleoside methylphosphonates are shown in Figure 1. Four types of targets were studied: two 19-mers designated d-I or r-I, which contain respectively a deoxyribo- or ribonucleotide backbone and two 29-mers designated d-II or r-II. The 19-mer targets are not self-complementary and are therefore expected to exist in essentially a random coil conformation. The 29-mers contain the same sequences found in d-I and r-I. They have self-complementary ends whose sequences are indicated by the shaded nucleotides, and they are therefore expected to form hairpin structures consisting of a 7-base pair stem and a 15-base loop. Nucleotides 1–28 or r-II correspond to nucleotides 156–183 of the M-protein messenger RNA of vesicular stomatitis virus (VSV) (Rose & Gallione, 1981).

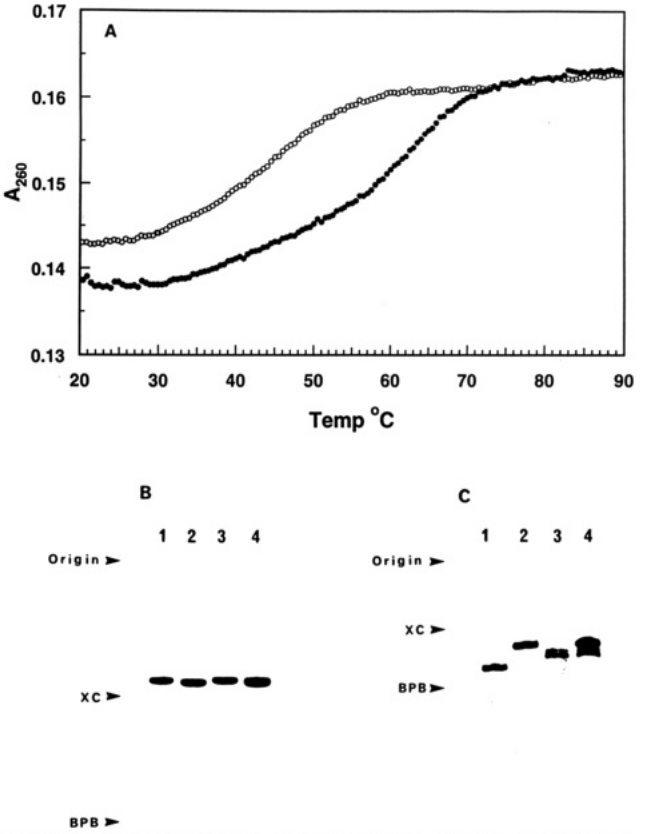

FIGURE 2: (A) Absorbance *vs* temperature profiles for d-II (○) and r-II (●) in 0.1 M sodium chloride/50 mM Tris at pH 7.6. Polyacrylamide gel electrophoresis under denaturing conditions (B) or nondenaturing conditions (C) of 0.1 μM [^{32}P]-d-II (lane 1), 0.1 μM [^{32}P]-d-III (lane 2), 0.1 μM [^{32}P]-d-IV (lane 3), and 0.1 μM [^{32}P]-d-III and 0.1 μM [^{32}P]-d-IV (lane 4). The arrows on the left side of the autoradiogram show the mobilities of xylene cyanol (XC) and bromophenol blue (PBP).

The complementary oligonucleoside methylphosphonates, oligomers A–D, contain a single phosphodiester linkage at the 5′-end of the oligomer. The oligomers were converted to their 5′-phosphates and further derivatized with 4′-[[*N*-(aminoethyl)amino]methyl]-4,5′,8-trimethylpsoralen as previously described (Miller, 1992). The psoralen-derivatized oligomers are designated as psA–psD. Oligomers A, C, and D are complementary to the loop regions of d-II and r-II, whereas the binding site for oligomer B includes the loop region and part of the stem of the hairpin targets.

The DNA and RNA target molecules were prepared by standard phosphoramidite methods (Brown & Brown, 1991; Scaringe *et al.*, 1990) and were characterized by chemical or enzymatic degradation. The structures of the targets were further characterized by UV absorbance *vs* temperature experiments, by chemical and enzymatic probing, and by gel mobility shift analysis. As expected, the A_{260} of d-I and r-I increased slightly in a linear manner over the temperature range 0–60 °C, behavior consistent with a random coil conformation. In contrast, as shown in Figure 2A, both d-II and r-II showed sigmoidal A_{260} *vs* temperature profiles. The melting temperature, T_m, for d-II is 43 °C in 0.1 M sodium chloride/50 mM Tris, pH 7.6, while that of r-II is 60 °C under the same buffer conditions. This behavior is consistent with the formation of hairpin structures by d-II and r-II.

The structure of d-II was further probed by digestion with S1 nuclease or by oxidation with potassium permanganate. When d-II was treated with S1 nuclease, very weak cleavage was seen at nucleotides G1 through A7 which comprise the 5′-side of the hairpin stem, and no cleavage was observed at

nucleotides T23 through C29 which comprise the 3′-side of the stem. In contrast, nucleotides in the hairpin loop were susceptible to S1 nuclease with very intense cleavage appearing after (3′-side) C12, A13, and A14. Much less cleavage was seen on the 3′-side of the loop.

Treatment of d-II with potassium permanganate, which oxidizes the 5,6 double bonds of pyrimidine bases, followed by chain cleavage with hot piperidine gave fairly strong cleavage of approximately the same intensity at T16, T19, T21, and T22. A moderate amount of reaction was seen at T8 and T9, whereas T3, T23, and T24, which occur in the stem, were not susceptible to oxidation by potassium permanganate. Reaction of the cytidines varied somewhat in individual experiments, but generally the cleavage was weak or nonexistent. In addition, cleavage was almost always seen at G5.

Target d-II could form an intermolecular duplex having two 7 base pair duplexes separated by a single-stranded internal loop, instead of an intramolecular hairpin. To rule out this possibility, gel mobility experiments were carried out on d-II and on the 29-mers d-III and d-IV. As shown in Figure 1, the seven bases at the 5′- and 3′-ends of d-III are complementary respectively to the seven bases at the 3′ and 5′-ends of d-IV. Thus, a 1:1 mixture of d-III and d-IV could form an intermolecular complex consisting of 2 duplex regions separated by an internal bubble of 15 nucleotides/strand. As shown in Figure 2B, oligomers d-II, d-III, and d-IV as well as a mixture of d-III and d-IV run as single bands of approximately the same mobility on a 20% denaturing polyacrylamide gel. However, as shown in Figure 2C, these oligomers have distinctly different mobilities on a 20% nondenaturing polyacrylamide gel. The mobility of d-II is faster relative to the mobility of either d-III or d-IV. This mobility behavior is consistent with a hairpin versus nonstructured single strands. A 1:1 mixture of d-III and d-IV gives two bands whose mobilities appear to be identical with those of the individual oligomers. Thus, it appears that these oligomers do not form an intermolecular duplex under these conditions. The structure of the RNA target, r-II, was probed using T_1 ribonuclease, which specifically cleaves on the 3′-side of single-stranded guanosines, or CL_3 ribonuclease, which cleaves at single-stranded cytosines. Under nondenaturing conditions in 0.1 M sodium chloride/50 mM Tris, pH 7.6, buffer, only G10 which is located on the 5′-side of the putative hairpin loop was cleaved by RNase T_1. Guanosines G1, G25, G26, and G28 located in the stem were resistant to cleavage. Likewise, nucleosides C12, C17, and C18 were cleaved by RNase CL_3, while no reaction was seen at C2, C4, C5, and C29.

Interactions between Oligonucleotide Targets and Their Complementary Oligonucleoside Methylphosphonates. Interactions between the linear targets, d-I and r-I, and the oligonucleoside methylphosphonates were examined by UV melting experiments. Figure 3 shows A_{260} *vs* temperature profiles for 1:1 mixtures of d-I and oligomer A or psA, and 1:1 mixtures of r-I and oligomer A or A′, an oligomer with the same sequence as A but with an all-phosphodiester backbone. Both A and psA form stable duplexes with DNA target d-I with T_ms of 35 and 34 °C, respectively. The melting temperatures of these duplexes are similar to that of the duplex formed between d-I and oligomer A′, whose T_m is 36 °C (data not shown). Phosphodiester oligomer A′ and RNA target r-I also form a stable duplex whose melting temperature is 29 °C. However, the A_{260} *vs* temperature profile of a 1:1 mixture of methylphosphonate oligomer A and the RNA target, r-I,

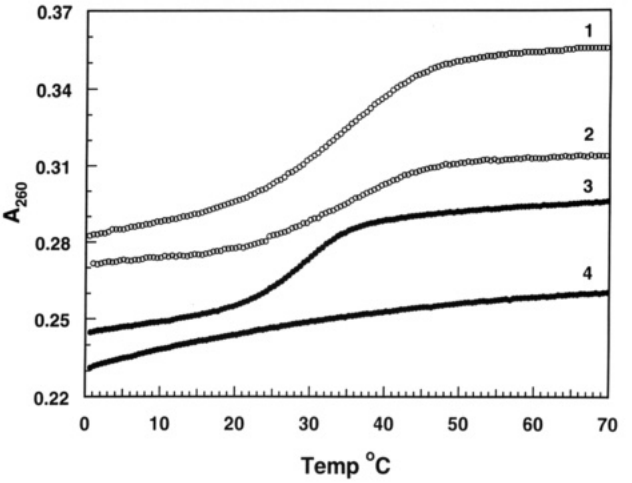

FIGURE 3: Absorbance *vs* temperature profiles for d-I/A (curve 1), d-I/psA (curve 2), r-IA′ (curve 3), and r-I/A (curve 4) in 0.1 M sodium chloride/50 mM Tris at pH 7.6. The total strand concentrations are 2.0 μM with the exception of d-I/A, which is 3.0 μM. The curves have been offset for clarity.

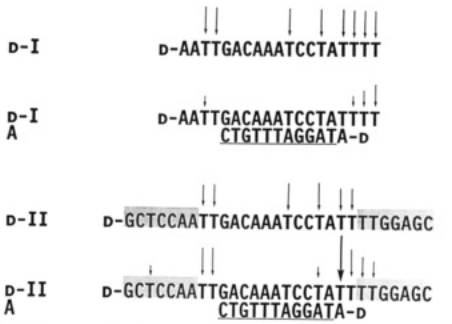

FIGURE 4: Effect of oligomer A on permanganate oxidation of thymidines in target oligomers d-I and d-II. Oxidation reactions were carried out on 0.1 μM target in the presence of 5 μM A in 70 μM potassium permanganate, 0.1 M NaCl, and 50 mM Tris, pH 7.6, at 20 °C for 30 min. The size of the arrows indicates the relative intensity of cleavage as analyzed by gel electrophoresis on a 20% polyacrylamide gel containing 7 M urea.

shows only a broad transition with no distinct inflection point over the temperature range 0–60 °C.

The interaction of oligomer B with DNA target d-I was also investigated (data not shown). Oligomer B can form an additional 4 base pairs with target d-I. However, the T_m of this duplex, 34 °C, was essentially the same as that of duplex d-I/A. The psoralen derivatives of B also formed a duplex, d-I/psB, whose T_m was 36 °C.

The site of interaction between oligomer A and DNA target d-I was characterized by footprinting experiments using permanganate oxidation. The results of these experiments are shown schematically in Figure 4. In the absence of oligomer A, all the thymidines of DNA target d-I are oxidized by permanganate to about the same extent when the reaction is carried out at 20 °C. In the presence of excess A, only T3, T17, T18, and T19 are oxidized. Binding of A to d-I protects T4, T11, and T14, nucleosides which are near or part of the binding site, from oxidation. This experiment provides direct evidence that oligomer A binds correctly to its complementary site on target d-I.

Thymidines in the loop region of hairpin target d-II are oxidized by permanganate at 20 °C. Oligomer A protects T16 and to a lesser extent T19 from oxidation. Surprisingly, T21, which is also part of the binding site of A, undergoes extensive oxidation. Thymidines T8, T9, and T22, which are located at the stem and loop junction of the hairpin, show

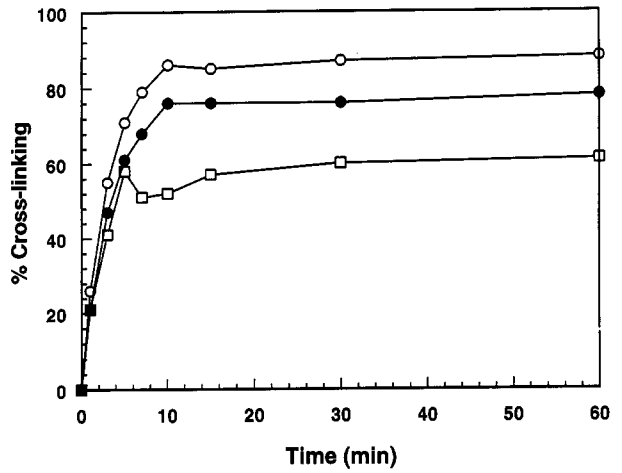

FIGURE 5: Kinetics of cross-linking between d-I/psA (○) and r-I/psA (●) in 0.1 M sodium chloride/50 mM Tris, pH 7.6, and d-II/psA (□) in 0.1 M sodium chloride, 20 mM magnesium chloride, and 50 mM Tris, pH 7.6, at 4 °C.

increased levels of oxidation in the presence of A as compared to the same nucleosides in d-I alone. Additional low levels of oxidation also occur at T3, T23, and T24, which are located in the stem-forming sequences of the hairpin.

Cross-Linking between Oligonucleotide Targets and Complementary Psoralen-Derivatized Oligonucleoside Methylphosphonates. Irradiation of duplexes formed between psoralen-derivatized oligonucleoside methylphosphonates and their complementary targets with 365 nm ultraviolet light results in the formation of a cyclobutane adduct between the pyrone ring of the psoralen and a pyridine base in the target molecule. In the cases of targets I and II, thymidines and uracils serve as the target sites for cross-linking. Recent experiments with duplex d-I/psA have shown that cross-links are formed at T17, T18, and T19 (Kean & Miller, 1993). Cross-linking reactions were carried out in 50 mM Tris buffer at pH 7.6 in the presence of 0.1 M sodium chloride using 0.1 μM target and 5.0 μM oligomer. The targets were 5′-end-labeled with a [^{32}P]phosphate group, and the cross-linking reactions were monitored by polyacrylamide gel electrophoresis under denaturing conditions.

Figure 5 shows the kinetics of cross-linking reactions for duplexes d-I/psA, r-I/psA, and d-II/psA at 4 °C. The reactions are essentially linear for the first 5 min and level off after 10 min. It appears that the initial rates of reaction are very similar for all three target molecules.

The effects of temperature on cross-linking are shown in Figure 6A–D. Both oligomers psA and psB cross-link to the extent of ~80% to the linear target d-I over the temperature range 4–40 °C as shown in Figure 6A. Cross-linking drops off rapidly above 40 °C, which is approximately 5 °C higher than the T_m for duplexes formed between the oligomers and d-I at a total strand concentration of 2 μM. Although cross-linking to the linear RNA target r-I is approximately as high as that observed with the DNA target at 4 °C, the cross-linking reaction is much more sensitive to increasing temperature. By 40 °C, essentially no cross-linking is observed for either oligomer.

As is shown in Figure 6B, oligomers psC and psD exhibit relatively rapid decreases in cross-linking as the temperature increases. Cross-linking by oligomer psD appears to be somewhat less sensitive to temperature than does cross-linking by the 8-mer psC. In contrast to their behavior with the DNA target, oligomers psC and psD showed very little cross-linking to the linear RNA target r-I, even at 4 °C.

Table 1: Cross-Linking between Psoralen-Derivatized Oligonucleoside Methylphosphonates and RNA Hairpin r-II

	% cross-linking[a]		
oligomer	5 μM	50 μM	100 μM
psA	4	37	nd
psB	4	nd	nd
psC	<1	2	4
psD	<1	16	26

[a] Cross-linking reactions were carried out in a buffer containing 0.1 M sodium chloride/50 mM Tris, pH 7.6, with 0.1 μM r-II and the indicated concentration of psoralen-derivatized oligomer at 4 °C. nd indicates cross-linking was not determined at this oligomer concentration.

Extensive cross-linking between the DNA hairpin target d-II and oligomers psA or psB was also observed as is shown in Figure 6C. The temperature above which cross-linking by the 12-mer psA begins to diminish, 25 °C, is approximately 5 °C lower than that observed for cross-linking by the 16-mer psB. In both cases, extensive cross-linking is observed well below the T_m of the hairpin target, 43 °C. Treatment of the photoadduct formed between oligomer psA and d-II with piperidine followed by gel electrophoresis gave a single major band corresponding to cleavage at T22 and fainter bands at T23 and T24. This result suggests that the primary site of cross-linking between oligomer psA and d-II is T-22 (Kean & Miller, 1993).

As was the case for the linear DNA target, cross-linking by oligomers psC or psD to the hairpin target was much less extensive and more sensitive to temperature than that observed for oligomers psA or psB. This is shown by the temperature profiles in Figure 6D. Both 8-mers, psC or psD, whose binding sites are within the single-stranded loop region of d-II, showed rapidly decreasing cross-linking with increasing temperature.

Although significant levels of cross-linking were observed between all four oligomers and DNA hairpin target d-II, very little cross-linking was observed between the oligomers and the RNA hairpin r-II, even at 4 °C. As indicated in Table 1, less than 5% cross-linking was observed when the reactions were carried out at an oligomer concentration of 5 μM. In the case of oligomers psA and psD, cross-linking increased significantly when the oligomer concentration was increased 10–20-fold. Both oligomers are complementary to the loop on the 3′-side of the hairpin. Oligomer psC, on the other hand, whose binding site is in the loop on the 5′-side of the hairpin, showed little cross-linking even at 100 μM concentration.

Cross-Linking between VSV mRNA and Oligomer psA. Oligomer psA is complementary to nucleotides 165–176 of VSV M-protein mRNA. The ability of this oligomer to cross-link to polyadenylated RNA isolated from VSV-infected mouse L-cells was examined *in vitro* (Levis, 1993). Cross-linking was carried out between 2 μg of VSV mRNA and 10 μM ^{32}P-labeled psA in a buffer containing 30 mM Tris-HCl, 20 mM magnesium chloride, and 300 mM potassium chloride at pH 6.0. As a control, the same reaction was carried out using polyadenylated RNA from uninfected mouse L-cells. The results of a cross-linking experiment carried out at 22 °C are shown in Figure 7. Cross-linking occurred specifically to M-protein mRNA in the experiment employing VSV mRNA. Essentially no cross-linking was observed when RNA from uninfected cells was used. The radioactivity at the top of the gel in both lanes represents non-cross-linked psA. The extent of cross-linking was estimated to be approximately 40% at this temperature.

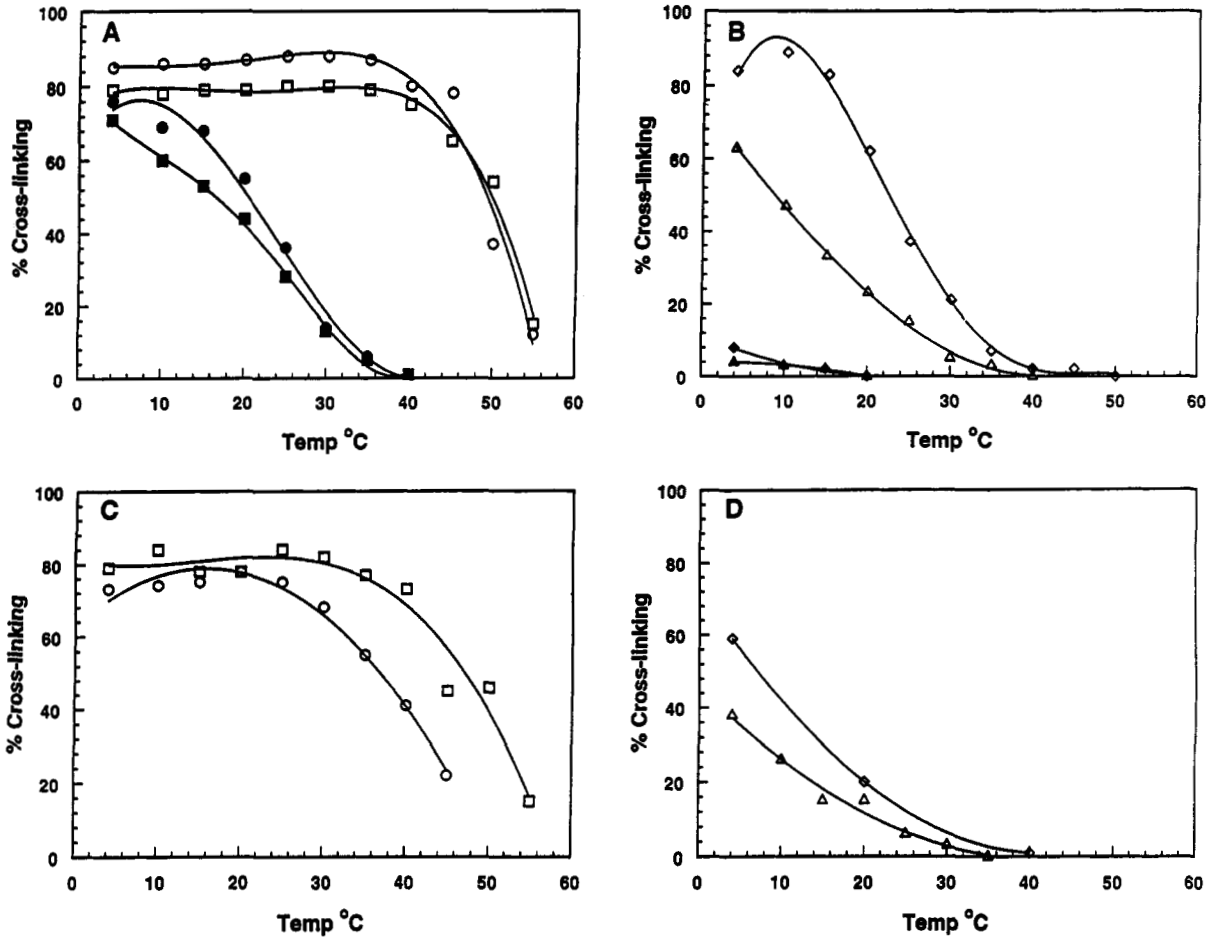

FIGURE 6: Effect of temperature on cross-linking between (A) d-I/psA (○), d-I/psB (□), r-I/psA (●), and r-I/psB (■); (B) d-I/psC (△), d-I/psD (◇), r-I/psC (▲), and r-I/psD (◆); (C) d-II/psA (○) and d-II/psB (□); and (d) d-II/psC (△) and d-II/psD (◇) in 0.1 M sodium chloride/50 mM Tris at pH 7.6.

DISCUSSION

We have investigated the possibility that cross-linking between psoralen-derivatized oligonucleoside methylphosphonates and complementary DNA or RNA target molecules can be affected by the structure of the target. The sequences of the 19-mer and 29-mer targets are based on a sequence, ...GCTCCAATTGACAAATCCTATTTTGGAG..., found in the coding region of VSV M-protein mRNA. Computer-assisted folding of VSV M-mRNA suggests that this sequence could adopt a hairpin structure having a loop of 15 nucleotides (Levis, 1993). The 19-mers d-I and r-I include this loop sequence. Although they contain two complementary bases at their 5′- and 3′-ends, d-I and r-I are expected to exist essentially as random coils in solution. This expectation is supported by thermal denaturation experiments in which only a small linear increase in the absorbance of d-I or r-I was observed with increasing temperature. Oxidation of d-I with potassium permanganate followed by treatment with hot aqueous piperidine gave essentially the same level of cleavage at all of the thymidine residues throughout the oligomer (Figure 4). This result suggests that none of these residues was protected by hydrogen bonding or extensive stacking interactions. Treatment of r-I with RNase also yielded a hydrolysis pattern consistent with a random coil structure. All three cytosines were cleaved by the single-strand cytosine-specific ribonuclease CL_3, and the only guanosine in the molecule was cleaved by RNase T_1 which recognizes single-stranded guanosines.

The 29-mers d-II and r-II showed behavior consistent with the formation of hairpin structures. Both oligomers displayed sigmoidal A_{260} *vs* temperature profiles (Figure 2A). The RNA hairpin was considerably more stable than the corresponding DNA hairpin. When probed with single-strand-specific chemical reagents or enzymes, both oligomers exhibited cleavage patterns consistent with a hairpin conformation. Thus, in the case of d-II, extensive cleavage at thymidines located in the loop was observed when the oligomer was treated with potassium permanganate and hot piperidine, whereas cleavage was not observed at thymidines in the self-complementary stem region. Nucleotides in the loop region of d-II were hydrolyzed by S1 nuclease whereas those in the stem underwent little or no hydrolysis. A similar sensitivity of G or C nucleotides in the loop region of r-II to cleavage by ribonuclease T_1 or CL_3 also corroborated the hairpin conformation of the RNA 29-mer. It appears unlikely that d-II exists as an intermolecular duplex rather than as a hairpin. When run under denaturing conditions, oligomer d-II and a 1:1 mixture of the partially complementary 29-mers d-III and d-IV each had similar polyacrylamide gel electrophoretic mobilities and thus similar structures (Figure 2B). Under nondenaturing conditions, a mixture of d-III and d-IV did not appear to form an intermolecular duplex, and the electrophoretic mobility of the hairpin, d-II, was, as expected, much greater than that of either d-III or d-IV (Figure 2C).

The 19-mer and 29-mer targets contain common binding sites for the psoralen-derivatized methylphosphonate oligomers (Figure 1). In each case, the psoralen group occurs opposite to a thymidine or uridine residue, respectively, in the complementary DNA or RNA target sequence. Oligomer A is targeted to a nucleotide sequence which occurs in the central

FIGURE 7: Cross-linking between psA and poly(A+) RNA from VSV-infected (lane 1) or uninfected (lane 2) mouse L-cells at 22 °C. The arrow on the left side of the autoradiogram shows the mobility of VSV M-protein mRNA as determined by ethidium bromide staining.

region of the 19-mer. This same nucleotide sequence comprises the major portion of the loop region of the 29-mer. The binding site for oligomer B includes the oligomer A binding site and extends to the 5′-end of the 19-mer or into the 5′-portion of the stem of the 29-mer. The binding site for oligomer C occurs on the 5′-side of the loop of the 29-mer, whereas the binding site for D is on the 3′-side of the loop.

The interactions of oligomer A and its psoralen derivative, psA, with the 19-mer and 29-mer targets were characterized by UV melting experiments and chemical footprinting experiments. As expected, both A and psA formed stable duplexes with DNA target d-I, whose T_ms are comparable with those of the parent diester, A′ (Figure 3). It appears that derivatization with psoralen does not significantly affect the ability of the methylphosphonate oligomer to interact with its complementary target, a result in agreement with previous studies (Lee *et al.*, 1988a). Similar UV melting experiments carried out with the hairpin DNA target d-II gave a profile with two transitions. The first, sharp sigmoidal transition occurred over the temperature range 12–26 °C with a midpoint at 18 °C. The second transition was very broad and occurred over the range 24–50 °C. Although the increased hypochromicity of d-II/A relative to that calculated for noninteracting strands suggests that d-II and A do form a complex, it is unclear what structural changes are represented by the observed transitions.

Evidence for the interaction of A with d-I or d-II was also provided by the chemical footprinting experiments (Figure 4). Permanganate oxidation of thymidine residues in d-I occurred in a uniform manner in the absence of oligomer A, whereas the presence of A resulted in protection of the thymidines associated with the oligomer binding site. This result is in agreement with the expectation that thymidines involved in base pair formation are protected from reaction with permangante (Hayatsu & Ukita, 1967). Recent experiments by Hansler and Rokita (1993) suggest that in addition to steric interference, increased electrostatic repulsion between permanganate and the backbone phosphate groups decrease the rate and extent to which the thymidines react in double-stranded *versus* single-stranded polynucleotides. It would appear in the case of d-I/A that steric interference is primarily responsible for the reduced reactivity of the thymidines at the oligomer binding site, because the nonionic methylphosphonate backbone of A should not significantly increase the negative charge density of the duplex.

Oligomer A also protects two of the thymidine residues, T16 and T19, in the oligomer binding site of DNA hairpin d-II from oxidation by permanganate. Disruption of base pairs in the stem must occur in order for oligomer A to form a duplex with nucleotides in the loop of d-II. This may account for the increased reactivity of T8, T9, T23, and T24 relative to their reactivity in the uncomplexed hairpin. The observation that T3 is only slightly reactive suggests that part of the stem remains intact upon oligomer binding. The hyperreactivity of T21 was unexpected and suggests that this thymidine residue is not hydrogen-bonded to the 5′-terminal A residue of oligomer A. Possibly the failure to completely disrupt the stem causes conformational changes at this position which more fully expose T21 to permanganate.

Although the melting profiles of 1:1 mixtures of oligomer A or psA and d-I show distinct transitions and reasonably high melting temperatures, the opposite behavior is observed for 1:1 mixtures of oligomer A and the RNA target r-I (Figure 3). It does not appear that the broad transition for r-I/A is due to a defective RNA target because r-I forms a stable duplex (T_m 29 °C) with oligodeoxyribonucleotide A′. The behavior observed with the DNA and RNA targets may result in part from the inherently lower stability of deoxyoligomer/RNA duplexes which exist in an A-type helix, *versus* deoxyoligomer/DNA duplexes which exist in a B-type helix, as seen in Figure 3 and observed by others (Inoue *et al.*, 1987). In addition, recent experiments have shown that the stabilities of duplexes formed between methylphosphonate oligomers and complementary DNA targets are influenced by the configuration of the methylphosphonate linkage and that the R_p configuration allows formation of more stable duplexes than does the S_p configuration (Lesnikowski *et al.*, 1990). The results shown in Figure 3 suggest that the duplexes formed between the DNA target and the various diastereoisomers of oligomer A have similar stabilities whereas those formed with the RNA target have a much broader range of stabilities. Thus, the configurations of contiguous methylphosphonate linkages may play a more important role in determining helix stability in DNA/RNA, A-type duplexes, than is the case in DNA/DNA, B-type duplexes.

The psoralen-derivatized oligomer psA cross-links with varying degrees of efficiency to both the linear and hairpin DNA and RNA targets. The rate of cross-linking appears to be essentially independent of the type of target backbone, RNA *vs* DNA, or the type of target structure, linear *vs* hairpin (Figure 5). The differences in the extent of cross-linking most likely reflect the different extents to which psA interacts with the various targets. The lack of further cross-linking after 10 min is due to the competing photodegradation of the psoralen group of unbound oligomer (Lee *et al.*, 1988a).

The extent of cross-linking between oligomer psA and its target is dependent upon its interaction with the target which in turn is influenced by the temperature at which the cross-linking reaction is carried out, the structure of the target, and the type of target backbone. The extensive cross-linking observed between psA and the linear DNA target d-I over the

temperature range 4–40 °C is consistent with the strong interaction of this oligomer with its target (Figure 6A). Similar behavior is exhibited by the 16-mer psB. The decrease in cross-linking above 40 °C is most likely due to dissociation of the target/oligomer duplex. It appears that dissociation takes place at temperatures which are somewhat higher than the T_ms of the target/oligomer duplexes observed in Figure 3. Under the conditions of the cross-linking experiment, the oligomer concentration is 5 μM, and the oligomer is in 50-fold excess of the target. Because the oligomer is in excess, a higher concentration of diastereoisomers with favorable R_p backbone configurations is available for binding with the target which could account for the higher apparent dissociation temperatures observed in these experiments.

Extensive cross-linking between psA or psB and the DNA hairpin target d-II was also observed (Figure 6C). In the case of psA, T22 was identified as the primary site of cross-linking. Thus, the psoralen group of psA is suitably positioned for cross-linking, despite the results from the footprinting experiment which suggested lack of base pair formation between the 5′-terminal A of the oligomer and T21 of the target. The psoralen group may help stabilize binding interactions between the 5′-end of the oligomer and the hairpin target.

In contrast to its behavior with the linear target d-I, cross-linking between psA and the DNA hairpin begins to diminish above 25 °C. This decrease most likely results from a lower association constant for the d-II/psA complex relative to that of the d-I/psA duplex. The effect of temperature on cross-linking between psB and the hairpin target, on the other hand, appears to be very similar to that of cross-linking with the linear target. This suggests that psB disrupts the stem structure d-II and that under these conditions the target behaves essentially as if it were a linear molecule.

Both psA and psB cross-link with the linear RNA target r-I. However, although the extent of cross-linking is comparable to that observed with the linear DNA target at 4 °C, the percent of cross-linking to r-I decreases dramatically as the temperature increases relative to that observed with d-I (Figure 6A). This decrease reflects the reduced stability of the methylphosphonate oligomers with the RNA target *versus* their stability with the DNA target and is consistent with the results of UV melting experiments. On the basis of the melting profile for r-I/A, one would have predicted little or no cross-linking between psA and r-I, even at 4 °C. The relatively high extent of cross-linking observed even at 20 °C most likely represents cross-linking between r-I and a subpopulation of diastereoisomers of psA or psB whose backbone configurations allow stable duplex formation between the oligomer and target.

In contrast to the high extent of cross-linking seen between psA or psB and DNA hairpin d-II, very little cross-linking was observed between these oligomers and RNA hairpin r-II (Table 1). This low level of cross-linking can be attributed to the inability of the methylphosphonate oligomers to effectively disrupt the highly stable stem of the RNA hairpin. Because binding to RNA targets is apparently quite sensitive to backbone configuration, it is not surprising that increasing the concentration of psA from 5 to 50 μM results in a 9-fold increase in the extent of cross-linking.

Cross-linking by psoralen-derivatized oligomers can be influenced by the structure and the sequence of the binding/cross-linking site. Oligomers psC and psD which are targeted to two different regions of d-I can both form duplexes consisting of six A-T base pairs and two G-C base pairs. The melting profiles of 1:1 mixtures of psC or psD and target d-I in 0.1 M sodium chloride/50 mM Tris buffer at pH 7.6 are identical with an estimated T_m of less than 50 °C under these conditions. Although the oligomers appear to form duplexes of equivalent stability, at any given temperature the extent of cross-linking of psD to target d-I is approximately twice that observed for psC (Figure 6B). This difference may be a consequence of the different sequences near the psoralen cross-linking site in the target. In the case of psC, the target nucleotide sequence near the 3′-end of the oligomer binding site is ...ATCCT..., where the underlined pyrimidines are adjacent to the oligomer binding site. Because cross-linking to cytosine appears to be less efficient than cross-linking to thymine or uracil (Kean *et al.*, 1988), the primary cross-linking site is expected to be T11. On the other hand, the partial binding sequence for psD is ...TTTT..., where again the underlined pyrimidines occur adjacent to the oligomer binding site. The psoralen group of psD could cross-link to either of the two thymidines outside the oligomer binding site (Kean & Miller, 1993) or could intercalate between the last two base pairs of the duplex and cross-link to the T residues of these base pairs (Lee *et al.*, 1988b). Thus, the psoralen group of psD has a potentially larger cross-linking target than does the psoralen group of psC, and this could account for the greater efficiency with which psD cross-links with d-I.

Oligomers psC and psD are complementary respectively to the 5′-side and 3′-side of the loop of DNA hairpin target d-II, and binding of either oligomer would result in formation of an 8 base pair duplex which is essentially coaxial with the stem of the hairpin. Because seven unpaired nucleotides remain, formation of this duplex would not require disruption of the stem. At 20 °C, the extent of cross-linking between psC and d-II is approximately the same as that seen for d-I/psC (Figure 6D). The most likely cross-linking site in d-II for the psoralen of psC, T16, is single-stranded and thus may be conformationally similar to the corresponding site in the linear target, d-I. In contrast, cross-linking between psD and the hairpin target at 20 °C is approximately 3 times less than that observed with the linear target. Unlike the situation in the d-I/psC duplex, the potential cross-linking sites for the psoralen group of psD in the d-II/psD complex are involved in base pairs. This requires the psoralen to form a completely intercalated complex in order to cross-link. This change in environment could account for the decrease in cross-linking observed for d-II/psD. Alternatively, oligomer binding to the 3′-side of the loop may be less stable than binding to the 5′-side of the loop.

Very low levels of cross-linking were observed between psC or psD and the linear or hairpin RNA targets, r-I or r-II (Figure 6B and Table 1). The extent of cross-linking between psD and r-II increased dramatically when the concentration of the oligomer was increased from 5 to 100 μM, suggesting that a subpopulation of diastereoisomers can effectively interact with this RNA hairpin target. A similar increase in the concentration of psC had essentially no effect on the level of cross-linking with r-II. This lack of cross-linking apparently results from an inability of the psC to form a stable complex with r-II even at low temperature. Thus, 100 μM psC had no protective effect when r-II was treated with RNase T_1, whereas cleavage of G5 in r-I was reduced by approximately 50% in the presence of 100 μM psC. The ability of psD to bind and cross-link to r-II and the apparent lack of significant binding by psC are in contrast to the results of Lima *et al.* (1992). They showed that a decaribonucleotide targeted to the 5′-side of a 16-nucleotide loop of a 47-mer hairpin target had an association constant of approximately 3×10^{10} M^{-1}

whereas a decaribonucleotide targeted to the 3'-side of the loop had an association constant of less than 1×10^5 M^{-1}.

The results of our experiments suggest that target structure and sequence can have a significant influence on binding/cross-linking reactions of psoralen-derivatized oligonucleoside methylphosphonates. It is interesting to note that oligomer psA which is complementary to the coding region of VSV M-protein mRNA was found to cross-link effectively and selectively to VSV M-mRNA *in vitro* at 20 °C (Figure 7). The extent of cross-linking was estimated to be approximately 40–50%, a level which is similar to that observed between psA and the linear RNA target r-I at this temperature. Although computer folding of the M-protein mRNA suggests that this binding site can exist as a part of a hairpin structure, the cross-linking experiment is consistent with a more open structure for this binding site. It is quite likely that the target structure will play an important role in the interaction of oligonucleotide analogs with mRNA, a molecule rich in secondary and tertiary structure. Methods which allow one to reliably predict such structures should be invaluable in assisting the rational design of antisense oligonucleotides.

ACKNOWLEDGMENT

We thank Dr. Joel T. Levis for carrying out the cross-linking experiments between oligomer psA and VSV mRNA.

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