Photochemical Cross-Linking of Psoralen-Derivatized Oligonucleoside Methylphosphonates to Rabbit Globin Messenger RNA†

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ABSTRACT: Antisense oligodeoxyribonucleoside methylphosphonates targeted against various regions of mRNA or precursor mRNA are selective inhibitors of mRNA expression both in cell-free systems and in cells in culture. The efficiency with which methylphosphonate oligomers interact with mRNA, and thus inhibit translation, can be considerably increased by introducing photoactivatable psoralen derivatives capable of cross-linking with the mRNA. Oligonucleoside methylphosphonates complementary to coding regions of rabbit α- or β-globin mRNA were derivatized with 4′-(aminoalkyl)-4,5′,8-trimethylpsoralens by attaching the psoralen group to the 5′ end of the oligomer via a nuclease-resistant phosphoramidate linkage. The distance between the psoralen group and the 5′ end of the oligomer can be adjusted by changing the number of methylene groups in the aminoalkyl linker arm. The psoralen-derivatized oligomers specifically cross-link to their complementary sequences on the targeted mRNA. For example, an oligomer complementary to nucleotides 56–67 of α-globin mRNA specifically cross-linked to α-globin mRNA upon irradiation of a solution of the oligomer and rabbit globin mRNA at 4 °C. Oligomers derivatized with 4′-[[*N*-(2-aminoethyl)amino]methyl]-4,5′,8-trimethylpsoralen gave the highest extent of cross-linking to mRNA. The extent of cross-linking was also determined by the chain length of the oligomer and the structure of the oligomer binding site. Oligomers complementary to regions of mRNA that are sensitive to hydrolysis by single-strand-specific nucleases cross-linked to an approximately 10–30-fold greater extent than oligomers complementary to regions that are insensitive to nuclease hydrolysis. Oligomers in which the psoralen group is opposite a uracil residue in the mRNA cross-linked to approximately a 10-fold higher extent than those with the psoralen group opposite a cytosine residue. Cross-linking of 5–20 μM psoralen-derivatized oligomers to globin mRNA results in specific inhibition of translation of the targeted mRNA to the extent of 40–60%, which is consistent with the extent of cross-linking of the oligomer. The ability of psoralen-derivatized oligonucleoside methylphosphonates to specifically cross-link with single-stranded regions of mRNA, the inhibitory effect of cross-linked oligomers on mRNA translation, and the relatively long half-lives of these oligomers in serum-containing media suggest that these nucleic acid analogues could be used to specifically control mRNA expression in living cells.

Antisense oligodeoxyribonucleotides and antisense oligodeoxyribonucleoside methylphosphonates targeted against various regions of mRNA or precursor mRNA have been found to be selective inhibitors of mRNA expression both in cell-free systems and in cells in culture (Stephenson & Zamecnik, 1978; Zamecnik & Stephenson, 1978; Blake et al., 1985a,b; Kawasaki, 1985; Agris et al., 1986; Cazenave et al., 1986; Cornelissen et al., 1986; Haeuptle et al., 1986; Smith et al., 1986; Toulme et al., 1986; Walder et al., 1986; Wickstrom et al., 1986; Zamecnik et al., 1986; Heikkila et al., 1987; Gupta et al., 1987; Lemaitre et al., 1987; Marcus-Sekura et al., 1987; Zerial et al., 1987; Shuttleworth & Colman, 1988). The effectiveness of inhibition by oligodeoxyribonucleotides is appreciably enhanced by the action of ribonuclease H like activities that are found in varying levels in many cell types as well as in cell-free translating systems (Minshull & Hunt, 1986; Cazenave et al., 1987a,b; Dash et al., 1987). This enzyme cleaves the RNA strand of the duplex formed between the oligodeoxyribonucleotide and mRNA. Because the oligomer remains intact, it is free to bind to another molecule of mRNA and initiate another round of cleavage. Thus, relatively low concentrations of oligomer can cause extensive degradation of the mRNA resulting in high levels of translation inhibition.

Oligonucleoside methylphosphonates, on the other hand, apparently do not function by causing RNase H catalyzed cleavage of mRNA. Rather, it appears that these nonionic oligonucleotide analogues inhibit translation by physically blocking ribosome binding at the initiation codon site or during the elongation step of protein synthesis. Thus, rather high oligomer concentrations, in the range of 30–200 μM, are required to achieve 50–80% inhibition (Blake et al., 1985b; Agris et al., 1986; Smith et al., 1986; Marcus-Sekura et al., 1987).

The efficiency with which methylphosphonate oligomers inhibit translation could be considerably increased if the oligomer were to bind irreversibly to the targeted nucleic acid. Such irreversible binding can in principle be achieved by derivatizing the oligomer with groups capable of covalent bond formation with the mRNA. Several groups have reported derivatizing oligonucleotides with alkylating agents or photoreactive groups capable of cross-linking the oligomer to its target nucleic acid upon binding (Vlassov et al., 1983, 1984, 1986; Knorre & Vlassov, 1985; Gorshkova et al., 1986; Webb & Matteucci, 1986; Doan et al., 1987a,b; Praseuth et al., 1988).

Recently we described the synthesis of (aminoalkyl)trimethylpsoralen-derivatized oligonucleoside methylphosphonates and our studies on their interaction with single-stranded DNA (Lee et al., 1988). Extensive sequence-specific cross-linking to single-stranded DNA was observed

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when a mixture of the DNA and a complementary oligomer was irradiated at 365 nm, a wavelength well above the absorbance range of the nucleic acid bases. In this paper we describe the interaction of psoralen-derivatized methylphosphonate oligomers with rabbit globin mRNA and their effect on mRNA translation. Our results demonstrate that these oligomers covalently bind to mRNA in a sequence-dependent manner and as a consequence inhibit mRNA translation at low oligomer concentrations.

Experimental Procedures

[γ-^{32}P]ATP was purchased from Amersham Inc., and [^{35}S]methionine was purchased from New England Nuclear, Inc. T4 polynucleotide kinase was purchased from United States Biochemical Corp., and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide was obtained from Sigma Chemical Co. Rabbit globin mRNA, rabbit reticulocyte lysate, and wheat germ extract were obtained from Bethesda Research Laboratories, Inc. TBE buffer contains 0.089 M Tris, 0.089 M boric acid, and 0.2 mM EDTA[1] (Maniatis et al., 1982). SSC is 0.15 M sodium chloride and 15 mM sodium citrate, pH 7.0 (Maniatis et al., 1982). Northern blots were performed on GeneScreen nylon filters purchased from New England Nuclear, Inc. Oligonucleotide hybridizations were carried out in chambers purchased from Hoefer Scientific Instruments with a buffer that contained 1× SSC, 0.2% poly(vinylpyrrolidone), 0.2% Ficoll, and 0.2% bovine serum albumin.

Synthesis of 4′-[[N-(Aminoalkyl)amino]methyl]-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 described previously (Lee et al., 1988). 4′-[[*N*-(6-Aminohexyl)amino]methyl]-4,5′,8-trimethylpsoralen [(ah)AMT] was prepared in a similar manner.

Synthesis and Phosphorylation of Oligodeoxyribonucleoside Methylphosphonates. Methylphosphonate oligomers were synthesized as previously described (Miller et al., 1986; Lee et al., 1988) and after purification were phosphorylated at the 5′-terminus. The reaction mixture, containing 0.05 M Tris-HCl (pH 7.6), 0.01 M magnesium chloride, 5 mM dithiothreitol, 90 μmol of oligonucleoside methylphosphonate, 2 mM ATP, and 200 units of T4 polynucleotide kinase in a total volume of 0.45 mL, was incubated for 40 min at 37 °C. To label the 5′ end with [^{32}P]phosphate, 450 pmol of methylphosphonate oligomer was incubated in 30 μL with 50–330 pmol of adenosine [γ-^{32}P]triphosphate (sp act. 50–500 Ci/mmol), 0.05 M Tris-HCl (pH 7.6), 0.01 M magnesium chloride, 5 mM dithiothreitol, 10 μM ATP, and 15 units of T4 polynucleotide kinase for 40 min at 37 °C.

Synthesis of 4′-[[N-(Aminoalkyl)amino]methyl]-4,5′,8-trimethylpsoralen Derivatives of Oligodeoxyribonucleoside Methylphosphonates. 4′-[[*N*-(Aminoalkyl)amino]methyl]-4,5′,8-trimethylpsoralen-derivatized oligomers were prepared by reacting the oligomer with the psoralen in the presence of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide for 3–24 h as previously described (Lee et al., 1988). In some cases the reaction mixtures were treated with 0.1 M NH_4HCO_3 for 1 h at 0 °C, in order to reduce side products. All the derivatized oligomers were purified by polyacrylamide gel electrophoresis on 15% gels containing 7 M urea.

Irradiation of d-AMT-pTpGCACCAT. Two solutions each containing 20 000 cpm of d-AMT-[^{32}P]pTpGCACCAT dissolved in 5 μL of water were irradiated at 365 nm for 60 min at 4 °C. One of the solutions was further irradiated at 254 nm for 30 min at 4 °C. The solutions were lyophilized, and the residues were subjected to polyacrylamide gel electrophoresis on a 20% gel containing 7 M urea. The wet gel was autoradiographed overnight at −80 °C.

Interaction of (ae)AMT-Derivatized Oligodeoxyribonucleoside Methylphosphonates with Rabbit Globin Messenger RNA. Solutions containing 0.33 μM rabbit globin mRNA and 0–5.0 μM ^{32}P-labeled (ae)AMT-derivatized oligodeoxyribonucleoside methylphosphonate in 12 μL of water were preincubated in borosilicate glass test tubes at 37 °C for 2 min. Each tube was irradiated for 16 min at 4 °C by a long-wavelength ultraviolet lamp (Ultraviolet Products Inc.) whose maximum output occurs at 365 nm. The borosilicate glass test tube does not transmit light below 300 nm. Under these conditions, the intensity of irradiation was 0.83 J cm^{-2} min^{-1} as measured by a Blak-Ray J-221 long-wavelength ultraviolet intensity meter. All irradiations of mRNA were carried out under these conditions. The reaction mixtures were lyophilized, dissolved in gel loading buffer, and subjected to either agarose or polyacrylamide gel electrophoresis. The 1.4% agarose gels (13 cm × 11 cm × 1 cm) containing 1.2 M formaldehyde were run at 100 V for 4 h at room temperature in running buffer containing 20 mM MOPS (pH 7.0), 5 mM sodium acetate, and 1 mM EDTA. The 7% polyacrylamide/0.25% agarose gels (16 cm × 14 cm × 0.75 cm) containing 7 M urea were run in TBE buffer for 4 h at 500 V. The wet gels were autoradiographed at −80 °C. In order to locate the positions of α- and β-globin mRNA on the polyacrylamide gels, lanes containing UV-irradiated mRNA were stained with ethidium bromide.

Northern Blot Analysis of Rabbit Globin mRNA Cross-Linked with (ae)AMT-Derivatized Oligodeoxyribonucleoside Methylphosphonates. Solutions containing 0.33 μM rabbit globin mRNA or 0.33 μM rabbit globin mRNA and 5.0 μM (ae)AMT-derivatized oligodeoxyribonucleoside methylphosphonate were irradiated at 365 nm. They were subjected to agarose gel electrophoresis as described above, and the gels were blotted onto a nylon filter (Ausubel et al., 1987). After being baked in a vacuum oven for 2 h at 80 °C, the filters were prehybridized in hybridization buffer for 30 min at room temperature and then incubated for 16 h at room temperature with 20 mL of hybridization buffer containing 5′-^{32}P-labeled oligodeoxyribonucleotide probe. The filter was then rinsed with 20 mL of hybridization buffer, washed for 10 min with 20 mL of 5× SSC, and finally washed twice for 15 min with 2× SSC before autoradiography at −80 °C.

Gel Filtration. Solutions containing 0.33 μM globin mRNA and 5.0 μM ^{32}P-labeled (ae)AMT oligomer (5–10 Ci/mmol) were irradiated as described above. The reaction mixtures were lyophilized, redissolved in 15 μL of loading buffer containing 20 mM HEPES, 100 mM potassium chloride, 3 mM magnesium acetate, and 50% glycerol, and run on a Sephadex G-100 column (1.2 × 12.8 cm) with running buffer containing 20 mM HEPES, 100 mM potassium chloride, and 3 mM magnesium acetate. The percentage of mRNA cross-linked with derivatized oligomer was determined by measuring the amount of radioactivity in each fraction.

Cell-Free Translation of Rabbit Globin mRNA Cross-Linked with (ae)AMT-Derivatized Oligodeoxyribonucleoside

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SSC, standard sodium citrate buffer; VSV, vesicular stomatitis virus.

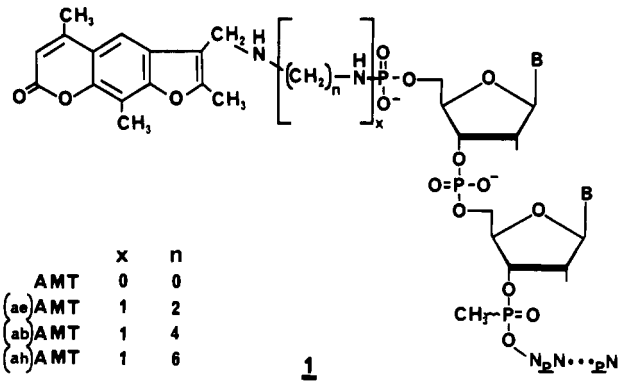

FIGURE 1: Structures of 4′-(aminoalkyl)-4,5′,8-trimethylpsoralen-derivatized oligodeoxyribonucleoside methylphosphonates. The symbol p indicates a methylphosphonate–internucleoside bond.

Methylphosphonates. Solutions containing 0.156 μM rabbit globin mRNA and 0–20 μM (ae)AMT-derivatized oligodeoxyribonucleoside methylphosphonate were irradiated as described above. The treated mRNA was used as a template for cell-free translation in either a rabbit reticulocyte lysate or a wheat germ extract. The commercial rabbit reticulocyte lysate, which had been pretreated with micrococcal nuclease (Pelham & Jackson, 1976) contained the following additional components: 3.5 mM magnesium chloride, 0.05 mM EDTA, 25 mM potassium chloride, 70 mM sodium chloride, 5 mM dithiothreitol, 25 μM hemin, 50 μg/mL creatine kinase, 1 mM calcium chloride, and 2 mM EGTA. Reactions, which were run in sterile, silanized glass test tubes, were initiated by the addition of the lysate. Each reaction mixture contained 25 mM HEPES (pH 7.2), 40 mM potassium chloride, 10 mM creatine phosphate, 90 mM potassium acetate, a 50 μM sample of each amino acid except methionine, 2.5 μCi of [^{35}S]-methionine, 0.14 μg of rabbit globin mRNA irradiated in the presence or absence of (ae)AMT-derivatized methylphosphonate oligomer, and 4.8 μL of rabbit reticulocyte lysate in a total volume of 14.8 μL. The reactions were incubated for 1 h at 37 °C.

The commercial wheat germ extract had also been pretreated with micrococcal nuclease and contained the following additional components: 20 mM HEPES (pH 7.5), 5 mM magnesium acetate, 100 mM potassium chloride, 5 mM 2-mercaptoethanol, 1 mM calcium chloride, and 2 mM EGTA. Reactions were initiated by the addition of the wheat germ extract and contained 20 mM HEPES (pH 7.5), 64 mM potassium acetate, 0.71 mM magnesium acetate, 2.3 mM adenosine triphosphate, 0.1 mM guanosine triphosphate, 5.5 mM creatine phosphate, 0.2 mg/mL creatine kinase, 80 μM spermidine phosphate, a 50 μM aliquot of each amino acid except methionine, 2.0 μCi of [^{35}S]methionine, 0.16 μg of rabbit globin mRNA irradiated in the presence or absence of (ae)AMT-derivatized methylphosphonate oligomer, and 4.8 μL of wheat germ extract in a total volume of 12.5 μL. Reactions were incubated for 1 h at 25 °C. The translation reactions were analyzed by gel electrophoresis as previously described (Blake et al., 1985a). Autoradiograms were scanned with an LKB Ultrascan XL laser densitometer to determine the relative intensities of the protein bands.

RESULTS

Psoralen-Derivatized Oligonucleoside Methylphosphonates Complementary to Globin mRNA. Figure 1 shows the general structure of the (aminoalkyl)trimethylpsoralen-derivatized methylphosphonate oligomers. All the internucleotide linkages consist of methylphosphonate groups, with the exception of the 5′-internucleotide bond, which is a phosphodiester. The oligomers are derivatized with either 4′-(aminomethyl)-4,5′,8-trimethylpsoralen (AMT), 4′-[[*N*-(2-aminoethyl)-amino]methyl]-4,5′,8-trimethylpsoralen [(ae)AMT], 4′-[[*N*-(4-aminobutyl)amino]methyl]-4,5′,8-trimethylpsoralen [(ab)AMT], or 4′-[[*N*-(6-aminohexyl)amino]methyl]-4,5′,8-trimethylpsoralen [(ah)AMT] through a 5′-phosphoramidate linkage (Lee et al., 1988).

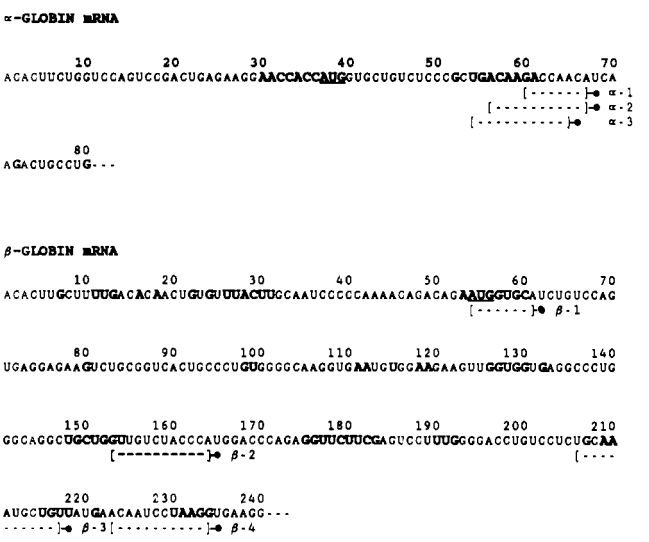

FIGURE 2: Binding sites on rabbit globin mRNA for psoralen-derivatized oligonucleoside methylphosphonates. The oligomer sequence is indicated below the mRNA sequence by [---]. The positions of the oligomer 3′- and 5′-terminal nucleosides are indicated respectively by the first and second brackets, and the position of the psoralen group is indicated by (●). The designation for each oligomer appears at the 5′ end of the oligomer. The phosphodiester bonds 3′ to the boldfaced nucleosides in the mRNA sequences are sensitive to cleavage by either T_1 or S1 nuclease as determined by Pavlakis et al. (1980).

As shown in Figure 2, the oligomers are complementary to the coding regions of rabbit α- or β-globin messenger RNA. For ease of reference the sequences of the methylphosphonate oligomers are designated by α or β followed by an Arabic number. The following oligomers complementary to rabbit α-globin mRNA were prepared: α-1, d-TpGTTGGTC; α-2, d-TpGTTGGTCTTGT; α-3, d-TpTGGTCTTGTCA. Methylphosphonate oligomers complementary to rabbit β-globin mRNA were also synthesized: β-1, d-TpGCACCAT; β-2, d-TpGGGTAGACAAC; β-3, d-ApCAGCATTTGCA; β-4, d-CpCTTAGGATTGT. The symbol "p" indicates a phosphodiester linkage, whereas underlined letters indicate the positions of methylphosphonate linkages. Psoralen-derivatized oligomers are prefixed with the symbol of the psoralen moiety.

The psoralen-derivatized methylphosphonate oligomers were prepared by reaction of 5′-phosphorylated oligonucleoside methylphosphonates with a 4′-(aminoalkyl)-4,5′,8-trimethylpsoralen in the presence of a water-soluble carbodiimide in 2,6-lutidine hydrochloride buffer (Lee et al., 1988). Since prolonged reaction with some methylphosphonate oligomer sequences resulted in the formation of undesired side products, the reaction time was limited to 3 h. Some of these side products could be converted to the desired psoralen-derivatized product by treatment with 0.1 M NH_4HCO_3 for 1 h at 0 °C. All the oligomers were purified by gel electrophoresis.

The phosphodiester and phosphoramidate linkages of the psoralen-derivatized oligomers are stable to hydrolysis in neutral aqueous solutions. The phosphodiester bond is slowly hydrolyzed by S1 nuclease, whereas the phosphoramidate linkage remains intact (Lee et al., 1988). d-AMT-[^{32}P]-

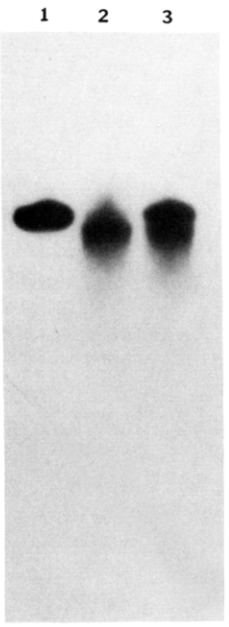

FIGURE 3: Intramolecular cross-linking of d-AMT-[^{32}P]-pTpGCACCAT. An aqueous solution of the oligomer was irradiated at 4 °C for 0 min at 365 nm (lane 1), 20 min at 365 nm (lane 2), or 20 min at 365 nm followed by 20 min at 254 nm (lane 3), and each solution was then subjected to PAGE as described under Experimental Procedures.

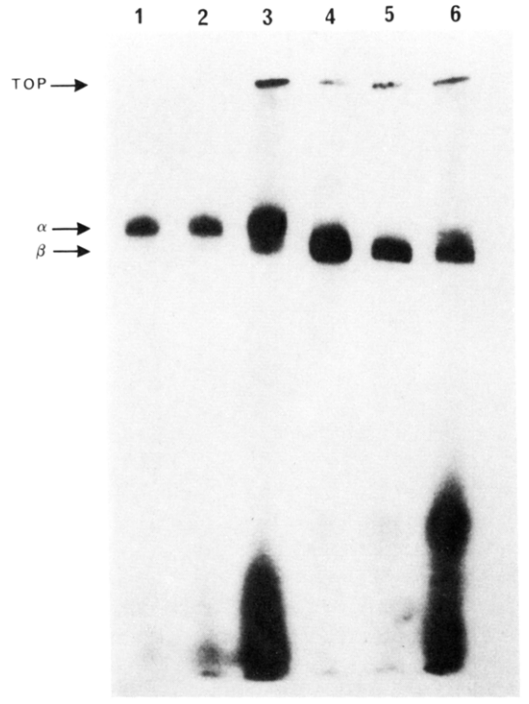

FIGURE 4: Cross-linking between 4′-[[*N*-(2-aminoethyl)amino]-methyl]-4,5′,8-trimethylpsoralen-derivatized oligonucleoside methylphosphonates and rabbit globin mRNA. Reaction mixtures containing 0.33 μM rabbit globin mRNA and 0.1 μM (lane 1), 1.0 μM (lane 2), and 5.0 μM (lane 3) [^{32}P](ae)AMT-α-2 or 0.1 μM (lane 4), 1.0 μM (lane 5), and 5.0 μM (lane 6) [^{32}P](ae)AMT-β-3 were irradiated for 16 min at 4 °C and then subjected to PAGE as described under Experimental Procedures. The specific activities of [^{32}P]-(ae)AMT-α-2 and [^{32}P](ae)AMT-β-3 were 150 Ci/mmol in lanes 1 and 4 and 15 Ci/mmol in lanes 2, 3, 5, and 6. The arrows on the left side of the autoradiogram show the mobilities of α- and β-globin mRNA as determined by ethidium bromide staining.

pTpGCACCAT was incubated at 37 °C in Casto's modification of Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum (Casto, 1973). Essentially no degradation of the oligomer was observed after 5 h as shown by polyacrylamide gel electrophoresis. Under these conditions, the half-life of this oligomer was determined to be approximately 48 h.

Psoralen-derivatized oligomers are cross-linked with nucleic acids by irradiation with 365-nm light (Lee et al., 1988). The effects of irradiation on the oligomers alone were studied. As shown in Figure 3, irradiation of d-AMT-[^{32}P]pTpGCACCAT at 365 nm yields a new product whose mobility on a polyacrylamide gel is greater than that of the original oligomer. Upon further irradiation of this material at 254 nm, an oligomer is produced whose mobility is identical with that of the starting oligomer. Although irradiation of d-(ae)AMT-[^{32}P]pTpGCACCAT at 365 nm gave a new product with greater mobility on a polyacrylamide gel, further irradiation at 254 nm did not result in conversion to the starting oligomer.

Photo-Cross-Linking with mRNA. The photo-cross-linking reactions were followed by using ^{32}P-labeled psoralen-derivatized methylphosphonate oligomers over a concentration range of 0.1–5.0 μM. A solution containing 0.33 μM α- and β-globin mRNA and the oligomer was irradiated at 365 nm, and the unreacted oligomer was separated from oligomer cross-linked to mRNA by either agarose or polyacrylamide gel electrophoresis. Photoadduct formation was demonstrated with agarose gels; however, under these conditions α- and β-globin mRNA comigrate (Murakami et al., 1986).

The cross-linking reaction was further characterized by polyacrylamide gel electrophoresis, which separates the two species of mRNA. A typical polyacrylamide gel is shown in Figure 4. For example, when a solution of [^{32}P](ae)AMT-α-2 and globin mRNA or [^{32}P](ae)AMT-β-3 and globin mRNA is irradiated at 365 nm for 16 min at 4 °C, radioactivity migrating with α-mRNA or β-mRNA, respectively, is observed. Likewise, specific photoadduct formation was observed between ^{32}P-labeled psoralen-derivatized α-1 or β-1 and their respective target mRNAs (data not shown). In the case of β-1, some cross-linking to α-globin mRNA was also observed. Seven of the eight nucleotides of β-1 are complementary to the initiation codon region of α-globin mRNA.

Cross-linking reactions between psoralen-derivatized oligomers and mRNA were also followed by Northern blot analysis. Solutions containing globin mRNA, (ae)AMT-α-2 and globin mRNA, or (ae)AMT-β-3 and globin mRNA were each irradiated at 365 nm. As a marker, [^{32}P](ae)AMT-β-3 was cross-linked with globin mRNA. The reaction mixtures were subjected to agarose gel electrophoresis, and the mRNA was transferred to a nylon filter by blotting. The portion of the filter containing the nonradioactive lanes was then probed with 5′-end-labeled d-[^{32}P]pACAGCATTTGCA, a diester oligomer whose nucleotide sequence is identical with that of (ae)AMT-β-3. As shown in Figure 5, the probe hybridizes to approximately the same extent with either globin mRNA or globin mRNA cross-linked with (ae)AMT-α-2, but much less extensively with globin mRNA cross-linked with (ae)-AMT-β-3. The mobility of the mRNA detected by the probe is identical with that of globin mRNA cross-linked with [^{32}P](ae)AMT-β-3.

The effect of linker length on the extent of cross-linking was examined. As a test system, oligomers with the nucleoside sequences d-TpGTTGGTC (α-1) or d-TpGTTGGTCTTGT (α-2) were derivatized with AMT, (ae)AMT, (ab)AMT, or (ah)AMT. The relative extents of cross-linking of the oligomers with α-globin mRNA were determined by polyacrylamide gel electrophoresis. The results are shown in Table I. The extent of cross-linking relative to that of AMT-α-1 increased approximately 6-fold when the psoralen was attached

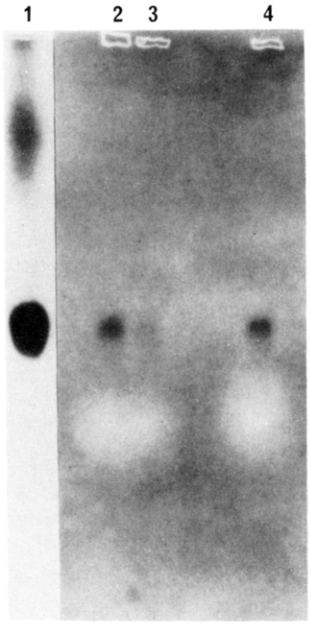

FIGURE 5: Northern blots of cross-linked products formed between 4′-[[*N*-(2-aminoethyl)amino]methyl]-4,5′,8-trimethylpsoralen-derivatized oligonucleoside methylphosphonates and rabbit globin mRNA. Lane 1 shows the product formed after 365-nm irradiation of 5.0 μM [^{32}P](ae)AMT-β-3 and 0.33 μM mRNA at 0 °C for 16 min. The blot containing lanes 2–4 was hybridized with ^{32}P-labeled d-pTCAGCATTTGCA, which is complementary to the β-3 binding site. Lane 2 contains the product formed after irradiation of 5.0 μM (ae)AMT-α-2 and 0.33 μM mRNA; lane 3 contains the product formed after irradiation of 5.0 M (ae)AMT-β-3 and 0.33 μM mRNA; lane 4 contains irradiated mRNA.

Table I: Effect of Linker Length and Oligomer Chain Length on Binding of (Aminoalkyl)psoralen-Derivatized Oligonucleoside Methylphosphonates to Rabbit Globin mRNA[a]

oligomer	relative binding
d-AMT-pTpGTTGGTC	1.0
d-(ae)AMT-pTpGTTGGTC	5.8
d-(ab)AMT-pTpGTTGGTC	4.0
d-(ah)AMT-pTpGTTGGTC	2.2
d-AMT-pTpGTTGGTCTTGT	6.5
d-(ae)AMT-pTpGTTGGTCTTGT	15.2

[a] Cross-linking reactions were carried out at 4 °C by irradiating an aqueous solution containing 0.01 μM psoralen-derivatized oligomer and 0.156 μM rabbit globin mRNA for 15 min at 365 nm.

via an aminoethyl linker. Further increase of the linker chain length resulted in diminishing extents of cross-linking. As expected, for a given linker length the efficiency of cross-linking increased as the chain length of the oligomer was increased.

The extent and specificity of cross-linking by (ae)AMT-derivatized methylphosphonate oligomers were critically dependent upon the sequences of the oligomers. Approximately 50–80% of β-globin mRNA was cross-linked with (ae)AMT-β-3 under the reaction conditions described in Figure 5 as determined by densitometric scanning of the autoradiograms of the Northern blots. Irradiation of (ae)AMT-α-2 with globin mRNA under similar conditions resulted in 50–100% cross-linking of α-globin mRNA as shown by Sephadex G-100 gel filtration column chromatography. In contrast to this behavior, (ae)AMT-α-3, whose nucleoside sequence is similar to that of (ae)AMT-α-2 but in which the psoralen moiety is positioned opposite a cytidine residue of the mRNA, showed approximately a 10-fold decrease in its extent of cross-linking to α-globin mRNA as assayed by polyacrylamide gel electrophoresis. A similar reduction in cross-linking was exhibited

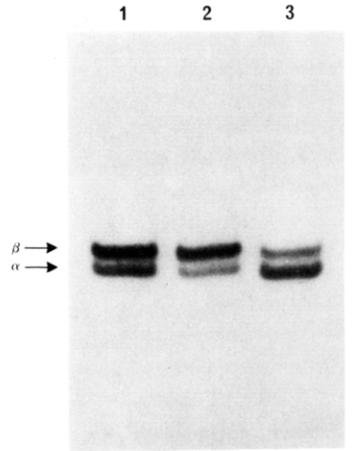

FIGURE 6: Effect of 4′-[[*N*-(2-aminoethyl)amino]methyl]-4,5′,8-trimethylpsoralen-derivatized oligonucleoside methylphosphonates cross-linked to rabbit globin mRNA on translation in a rabbit reticulocyte lysate. Aqueous solutions containing 0.33 μM globin mRNA were irradiated at 4 °C for 16 min at 365 nm in the presence of 0 μM oligomer (lane 1), 5.0 μM (ae)AMT-α-2 (lane 2), or 5.0 μM (ae)AMT-β-3 (lane 3). The mRNA from each reaction mixture was then translated in a rabbit reticulocyte lysate at 37 °C, and the reaction products were subjected to SDS–PAGE as described under Experimental Procedures. The mobilities of α- and β-globin are shown by the arrows at the side of the autoradiogram.

by (ae)AMT-β-2 and by (ae)AMT-β-4. The binding sites of these oligomers are to different areas of the coding region of β-globin mRNA. Although (ae)AMT-β-4 cross-linked specifically with β-globin mRNA, (ae)AMT-β-2 cross-linked to both α- and β-globin mRNA in a ratio of approximately 3:1. Irradiation of globin mRNA and d-(ae)AMT-[^{32}P]pCpAGACATTTTGA, an oligomer complementary to the initiation codon region of VSV N protein mRNA, resulted in no photoadduct formation.

Effect of Cross-Linking on Cell-Free Translation of Globin mRNA. The effects of cross-linking on in vitro translation of globin mRNA in a rabbit reticulocyte lysate and in a wheat germ extract were determined. Solutions containing 0.33 μM rabbit globin mRNA and varying concentrations of (ae)-AMT-α-2 or (ae)AMT-β-3 were irradiated at 4 °C, and the mRNA was then translated in a rabbit reticulocyte lysate at 37 °C or in a wheat germ extract at 25 °C. The [^{35}S]-methionine-labeled proteins were analyzed on polyacrylamide gels as shown in Figure 6 and the autoradiograms were scanned by a densitometer to determine the relative amounts of α- and β-globin that were synthesized.

As shown in Table II, (ae)AMT-α-2, which cross-links specifically to α-globin mRNA, specifically inhibits synthesis of α-globin. Likewise, similar specific translation inhibition is observed for (ae)AMT-β-3, which cross-links specifically to β-globin mRNA. No inhibition was observed when mRNA was irradiated in the presence of (ae)AMTpCpAGACATTTTGA, which is complementary to the initiation codon region of VSV N protein mRNA and which was shown not to cross-link to either α- or β-globin mRNA.

Essentially no inhibition of translation was observed when mRNA was irradiated with oligomers at concentrations of 1 μM or less. At oligomer concentrations greater than 10 μM, the amount of nonspecific translation inhibition increased whereas the amount of specific inhibition remained about the same. No inhibition of translation was observed when the oligomers were simply added to the mRNA without irradiation. Decreasing the time of irradiation resulted in less translation inhibition, whereas irradiating for longer than 16 min did not lead to increased inhibition of protein synthesis.

Table II: Inhibition of Translation of Rabbit Globin mRNA Cross-Linked with (Aminoethyl)trimethylpsoralen-Derivatized Oligonucleoside Methylphosphonates

oligomer	[oligomer] during irradiation[a] (μM)	% inhibition[b] reticulocyte[c] α	reticulocyte[c] β	wheat germ[d] α	wheat germ[d] β
d-(ae)AMT-pTpGTTGGTCTTGT	5	43	8	46	16
[(ae)AMT-α-2]	10	25	5	45	19
	20	38	26	70	40
d-(ae)AMT-pApCAGCATTTGCA	5	13	67	30	75
[(ae)AMT-β-3]	10	2	67	27	67
	20	2	64	50	76
d-(ae)AMT-pCpAGACATTTTGA[e]	5	0	0	0	0

[a] Cross-linking reactions were carried out at 4 °C by irradiating an aqueous solution containing the psoralen-derivatized oligomer and 0.156 μM rabbit globin mRNA for 16 min at 365 nm. These solutions were diluted by a factor of 3.3 or 2.5 prior to translation in the rabbit reticulocyte lysate or wheat germ extract, respectively. [b] The results are the average of two to five determinations. [c] Translation reactions were carried out at 37 °C. [d] Translation reactions were carried out at 25 °C. [e] This oligomer is complementary to the initiation codon region of VSV N protein mRNA.

Discussion

In a previous paper we described the photoinduced cross-linking of psoralen-derivatized oligonucleoside methylphosphonates with complementary regions of single-stranded DNA (Lee et al., 1988). This system can be easily manipulated and allows one to study, for example, the kinetics, the effect of temperature, and the effect of oligomer concentration on the cross-linking reaction. It appears, however, that the most promising targets for antisense oligonucleotides are mRNA and its unspliced precursor (Miller & Ts'o, 1988). Thus, in order to investigate these interactions with mRNA, we have prepared psoralen-derivatized oligonucleoside methylphosphonates with sequences complementary to the coding regions of rabbit α- or β-globin mRNA.

The interaction of oligonucleoside methylphosphonates with rabbit globin mRNA has been previously characterized (Murakami et al., 1985). These studies showed that oligonucleoside methylphosphonates complementary to the initiation codon region or the coding regions of rabbit globin mRNA bind in a specific manner to their target site on the mRNA. As a consequence of binding, translation of globin mRNA in both cell-free systems and in rabbit reticulocytes is inhibited (Blake et al., 1985b).

The psoralen-derivatized oligomers, whose structures are shown schematically in Figure 1, contain methylphosphonate internucleoside linkages having R_P and S_P configurations. Because it is not possible to separate isomers at each step during the synthesis, each oligomer preparation contains 2^n diastereoisomers, where n is the number of methylphosphonate bonds.

The 5′-terminal internucleoside linkage consists of a phosphodiester group, and the (aminoalkyl)trimethylpsoralen moiety is attached to the 5′ end of the oligomer via a phosphoramidate linkage. Thus, the psoralen-derivatized oligomer has a net charge of −2 at neutral pH. The distance between the trimethylpsoralen and the 5′ end of the oligomer can be varied by changing the number of methylene groups in the connecting aminoalkyl linker arm.

Methylphosphonate oligomers derivatized with psoralen are stable to hydrolysis when stored in aqueous solutions at neutral pH. The phosphoramidate linkage and the methylphosphonate linkages are resistant to cleavage by endonucleases such as S1 nuclease. The phosphodiester linkage is slowly cleaved by S1 nuclease or by nuclease activity found in fetal bovine serum. It appears that the oligomer has a half-life of 48 h when incubated with serum-containing cell culture medium. Thus, the oligomers would appear to be sufficiently stable for use in experiments involving cells growing in culture.

Cross-linking reactions between psoralen-derivatized oligomers and globin mRNA were initially carried out with psoralen-derivatized α-1, α-2, β-1, and β-3, whose binding sites are shown in Figure 2. Three factors were taken into account when the sequences of these oligomers were designed. The first was the structure of the oligomer binding sites. As shown in Figure 2, the targeted regions of the mRNAs are susceptible to cleavage by RNase T_1 and/or S1 nuclease and thus should be single-stranded and available for oligomer binding (Pavlakis et al., 1980). Although only a portion of the target region for α-1 and α-2 is sensitive to single-strand-specific nucleases, the entire region encompassing nucleotides 53–73 appears as a single-stranded loop in a computer-generated secondary structure model of rabbit α-globin mRNA (Pavlakis et al., 1980). Secondly, the 5′-psoralen groups of these oligomers oppose a uridine in the mRNA. Previous studies have shown that derivatives of psoralen specifically undergo cycloaddition reactions with pyrimidine bases and preferentially form photoadducts with thymine and uracil residues of nucleic acids (Cimino et al., 1985). Finally, a computer search showed that each oligomer should specifically form only one full-length duplex with its targeted mRNA and none with the nontargeted mRNA.

Cross-linking of the ^{32}P-labeled psoralen-derivatized α-1, α-2, β-1, and β-3 to mRNA was readily followed by polyacrylamide gel electrophoresis, an example of which is shown in Figure 4. Each of these oligomers cross-linked to its targeted mRNA in a specific manner. For a given oligomer sequence, no change in cross-linking specificity was observed when the oligomer was derivatized with AMT, (ae)AMT, (ab)AMT, or (ah)AMT. No cross-linking was observed with a noncomplementary oligomer d-(ae)AMT-[^{32}P]pCpAGACATTTTGA. It appears that the oligomers specifically cross-link to their complementary nucleotide sequences on the mRNA. This was demonstrated by hybridization of the oligodeoxyribonucleotide probe d-ACAGCATTTGCA, whose sequence and thus mRNA binding site are the same as β-3, to Northern blots of mRNA that had been cross-linked with either α-2 or β-3. As shown in Figure 5, the probe hybridized equally well to mRNA or to mRNA cross-linked with α-2 but not to mRNA cross-linked with β-3. Thus, the cross-linked β-3 effectively blocked hybridization of the probe to its binding site on β-mRNA. Taken together, these results suggest that the specificity of the cross-linking reaction is dictated primarily by the binding of the oligomer to its complementary nucleotide sequence on the mRNA and that the specificity of oligomer binding is not impaired by derivatization with psoralen. A similar conclusion was reached from studies involving cross-linking of psoralen-derivatized oligonucleoside methylphosphonates with single-stranded DNA (Lee et al., 1988).

The extent of cross-linking of the psoralen-derivatized oligomers to mRNA depends upon the temperature of the cross-linking reaction, the concentration of the oligomer, the length of the aminoalkyl linker arm, the chain length of the oligomer, and the nature of the oligomer binding site. In the present studies the cross-linking reactions were carried out at 4 °C in order to maximize oligomer binding and to limit potential nuclease degradation of the mRNA.

Cross-linking of ^{32}P-labeled oligomers could be detected at oligomer concentrations as low as 0.01 μM. Under these

conditions in which the concentration of either α- or β-mRNA was in approximately 7.5-fold excess of that of the oligomer, the extent of cross-linking was estimated to be 1% or less. The greatest extent of cross-linking was observed for oligomers derivatized with (ae)AMT as shown by the data in Table I. Examination of molecular models shows that the aminoethyl linker arm can position the psoralen in the optimal orientation for cross-linking to the target uracil residue in the mRNA. Although the longer aminobutyl and aminohexyl linkers also allow the psoralen to reach the uracil residue, the greater flexibility of these linkers must reduce their cross-linking efficiencies due to entropic effects. It is interesting to note that a similar dependence of cross-linking efficiency on linker arm length was observed for oligomers targeted against single-stranded DNA (Lee et al., 1988). The increased extent of cross-linking of AMT- or (ae)AMT-α-2, a dodecamer, versus that of AMT- or (ae)AMT-α-1, an octamer, is consistent with the expectation that the stability of the duplex formed by the oligomer and the mRNA should increase with increasing chain length of the oligomer.

The extent of cross-linking of (ae)AMT-α-2 or (ae)AMT-β-3 was estimated to be between 50 and 100% at oligomer concentrations of 5 μM. Under these conditions, the oligomer was in 32-fold excess of either α- or β-globin mRNA. The amount of mRNA cross-linked by the oligomer was assayed by probing Northern blots of mRNA cross-linked with unlabeled oligomer or by Sephadex G-100 gel filtration chromatography. Although the irradiation reactions were carried out under identical conditions, a considerable amount of variability in the extent of cross-linking as determined by either assay procedure was observed. This may reflect the inherent variability of the assay techniques. In any event it appears that rather extensive cross-linking can be achieved by using the (ae)AMT-derivatized methylphosphonate oligomers.

The effect of the structure of the mRNA binding site on photoadduct formation by (ae)AMT-derivatized methylphosphonate oligomers was investigated. For these experiments, two (ae)AMT-derivatized oligomers complementary to β-globin mRNA were used. In both cases the photoreactive psoralen is opposite a uracil residue in the mRNA. As shown in Figure 2, (ae)AMT-β-2 is targeted to a region of the mRNA that is relatively insensitive to cleavage by single-strand-specific nucleases; the only sites of cleavage are opposite the two 3′-terminal bases of the oligomer. The sequence of (ae)AMT-β-4 was chosen such that its psoralen-linked 5′-terminus could base pair with five bases of the mRNA that are susceptible to RNase T_1 or S1 nuclease cleavage. The remaining seven nucleotides of the binding site for the 3′-terminus of the oligomer are not cleaved. Although the resistance to nuclease cleavage in the regions is consistent with base-paired secondary structure, computer-generated secondary structure models do not extend to either of these two regions. It should also be realized that a site in the mRNA that is resistant to cleavage by single-strand-specific nucleases may not necessarily be involved in base pairing; tertiary structure may also account for the lack of cleavage.

Cross-linking with [^{32}P](ae)AMT-β-4 was specific for β-globin mRNA, although the extent of cross-linking was approximately 10-fold less than that of [^{32}P](ae)AMT-β-3. Oligomer [^{32}P](ae)AMT-β-2 cross-linked to β-mRNA about 30-fold less than [^{32}P](ae)AMT-β-3. Surprisingly, [^{32}P]-(ae)AMT-β-2 also cross-linked to α-globin mRNA although again the extent of cross-linking was about 10-fold less than that of [^{32}P](ae)AMT-β-2 to β-mRNA. A computer search for complementarity between β-2 and α-mRNA revealed three sites where the oligomer could form a partial duplex of five or more contiguous base pairs with the α-mRNA. Unfortunately, the globin mRNA structure mapping data of Pavlakis et al. (1980) does not extend to two of these sites: nucleotides 177–188, which have five contiguous complementary bases, and nucleotides 276–287, which have nine contiguous complementary bases. The psoralen moiety would be opposite a cytidine residue at both of these binding sites, a factor that would be expected to diminish the extent of cross-linking. The third potential binding site includes nucleotides 42–53, and the psoralen opposes a uracil residue in the mRNA. According to the secondary structure model, this region should be base paired in the mRNA and thus should be less accessible to oligomer binding.

Photo-cross-linking by [^{32}P](ae)AMT-α-3, which is complementary to nucleotides 54–65 of α-mRNA and whose psoralen group is opposite a cytidine, was approximately 10-fold less than that of [^{32}P](ae)AMT-α-2, which is complementary to nucleotides 56–67 and whose psoralen group is opposite a uridine in the mRNA. Because both dodecamers are targeted to essentially the same single-stranded region in α-globin mRNA and can form duplexes containing seven A-U base pairs and five G-C base pairs, they are expected to form duplexes of the same stability with α-mRNA. Therefore, the observed difference in the extent of cross-linking is most likely due to the base opposite the 5′-psoralen group, with cross-linking to uridine being more favorable than cross-linking to cytidine. This expectation is in agreement with work carried out by Bachellerie et al. (1981) in which they observed that 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen cross-linked more extensively to poly(U) than to poly(C) by a factor of 15 to 1. The results of these experiments and those described above suggest the extent of photoadduct formation by the psoralen-derivatized oligomers can be strongly influenced by the structure of the mRNA target site, and thus, if possible, these factors should be considered when psoralen-derivatized oligomers are designed for biological studies.

In addition to intermolecular cross-linking, it appears that some oligomers which terminate with a 5′-thymidine undergo intramolecular cross-linking. This is shown by the experiment described in Figure 3 in which the new product formed by irradiation of the AMT-derivatized oligomer at 365 nm is converted to the starting oligomer upon irradiation at 254 nm. This behavior is consistent with the formation of a cyclobutane bridge between the AMT moiety and the 5′-thymidine residue of the oligomer, although rigorous proof of structure remains to be carried out. Examination of molecular models shows AMT can stack upon the thymine ring of the 5′-terminal thymidine of the oligomer. The psoralen ring can lie parallel to either the 5′ or 3′ face of the thymine. As shown in the upper stereo drawing of Figure 7, when AMT is oriented parallel to the 5′ face, the 4′,5′ double bond of the furan ring lies below and is approximately parallel to the 5,6 double bond of the thymine ring. Upon irradiation, an intramolecular cycloaddition reaction could occur to form a cyclobutane bridge between the AMT furan ring and the thymine ring. As shown in the lower stereo drawing in Figure 7, the resulting cyclobutane adduct would have a cis–syn configuration. This configuration is found for the furan-side adducts formed by 8-methoxytrimethylpsoralen or by 4′-(hydroxymethyl)trimethylpsoralen and thymine bases of DNA (Kanne et al., 1982). In analogy with cross-links formed by AMT and DNA, this intramolecular cross-link would be expected to be reversed upon irradiation at 254 nm (Cimino et al., 1986; Shi & Hearst, 1987). When the AMT group is oriented parallel to the 3′

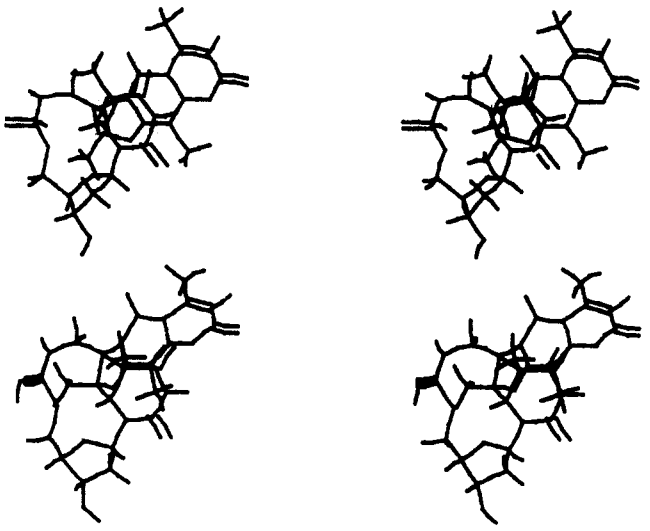

FIGURE 7: Stereo drawings of d-AMTpT (upper drawing) and the intramolecular cyclobutane adduct of d-AMTpT (lower drawing).

face of the thymine, the furan 4',5' double bond is located above and approximately perpendicular to the 5,6 double bond of the thymine. In this orientation, photocycloaddition appears less likely and would result in the formation of a highly strained cyclobutane bridge.

Although AMT-derivatized oligomers terminating in thymidine undergo intramolecular cross-linking, a similar reaction with (ae)AMT-derivatized oligomers apparently does not occur. The new product, which is observed after irradiation at 365 nm but which is not converted to starting material upon irradiation at 254 nm, may correspond to oligomer in which the pyrone ring of (ae)AMT has been opened (Hearst, 1981). As described by Lee et al. (1988), such ring-opened oligomer can no longer undergo photocycloaddition reactions. Although computer-generated models show the (ae)AMT group and the oligomer 5'-terminal thymidine could assume a stacked conformation in which the double bonds of the furan and thymine are aligned for cycloaddition, this apparently does not occur. Possibly the longer aminoethyl linker arm requires the (ae)-AMT to move away from the thymine ring to give a less stacked and more extended conformation than occurs in AMT-derivatized oligomers. In this extended form the furan and thymine double bonds would not be properly aligned and intramolecular cycloaddition would not occur. As described above, the formation of an extended conformation is consistent with the greater extent of cross-linking exhibited by (ae)-AMT-derivatized oligomers compared to those derivatized with AMT.

Cross-linking of psoralen-derivatized oligonucleoside methylphosphonates to mRNA would be expected to inhibit translation of the mRNA. The results of the experiments described in Table II are consistent with this expectation. The specificity and extent of inhibition observed in these reactions reflect the cross-linking behavior of the oligomers. For example, (ae)AMT-α-2 specifically cross-links with α-globin mRNA and specifically inhibits translation of this mRNA approximately 40% both in a rabbit reticulocyte lysate and in a wheat germ extract. As previously described, much higher concentrations of underivatized oligonucleoside methylphosphonates, 100–200 μM, are required to achieve comparable levels of translation inhibition in cell-free extracts (Blake et al., 1985b; Agris et al., 1986).

Irradiation of globin mRNA alone or the presence of 5 μM non-cross-linked, psoralen-derivatized oligomer in the translation reaction has essentially no inhibitory effect on translation. Similarly, irradiation of globin mRNA in the presence of a noncomplementary psoralen-derivatized dodecamer, (ae)AMT-pCpAGACATTTTGA, had no effect on translation. These results show that in the presence of low concentrations of oligomer the conditions for photo-cross-linking do not adversely effect mRNA and that low concentrations of psoralen-derivatized oligomers do not nonspecifically inhibit components of the translation reaction.

The ability of psoralen-derivatized oligonucleoside methylphosphonates to specifically cross-link with single-stranded regions of mRNA, the inhibitory effect of cross-linked oligomers on mRNA translation, and the relatively long half-lives of these oligomers in serum-containing media suggest that these nucleic acid analogues could be used to specifically control mRNA expression in living cells.

Registry No. d-AMT-pTpGTTGGTC, 117021-49-5; d-(ae)-AMT-pTpGTTGGTC, 117021-50-8; d-(ab)AMT-pTpGTTGGTC, 117021-51-9; d-(ah)AMT-pTpGTTGGTC, 117021-52-0; d-AMT-pTpGTTGGTCTTGT, 117067-36-4; d-(ae)AMT-pTpGTTGGTCTTGT, 117067-39-7; d-(ae)AMT-pCpAGACATTTTGA, 117067-38-6.

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