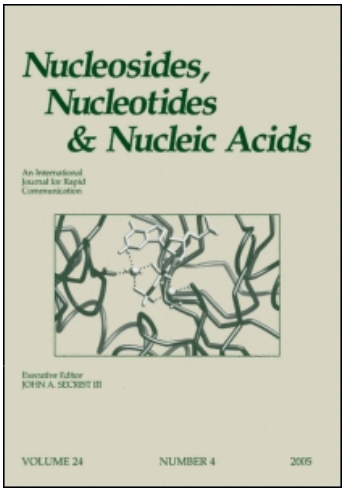

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Effects of Psoralen-Derivatized Oligonucleoside Methylphosphonates on Vesicular Stomatitis Virus (VSV) Protein Synthesis *In Vitro* and in VSV-Infected Cells

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EFFECTS OF PSORALEN-DERIVATIZED OLIGONUCLEOSIDE METHYLPHOSPHONATES ON VESICULAR STOMATITIS VIRUS (VSV) PROTEIN SYNTHESIS *IN VITRO* AND IN VSV-INFECTED CELLS†

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ABSTRACT: Oligodeoxyribonucleoside methylphosphonates (16-mers) targeted to VSV mRNAs were derivatized with 4'-{[N-(aminoethyl)amino]methyl}-4,5'8-trimethylpsoralen. These oligomers specifically inhibit translation of their targeted mRNAs *in vitro* following UV irradiation of the oligomer-mRNA complexes. Psoralen-derivatized oligonucleoside methylphosphonates are stable in cells and can inhibit VSV protein synthesis in culture following UV-irradiation of VSV-infected cells.

INTRODUCTION

In order for antisense oligonucleotides to specifically and effectively inhibit gene expression in cells, they must meet the following criteria. First, the oligonucleotides must specifically interact with their nucleic acid targets. Second, the oligomers must be resistant to cellular nucleases, and finally, the oligonucleotide reagents must be taken up by cells. The requirement of specificity and cellular stability are particularly critical for oligomers conjugated to functional groups capable of either cleaving or cross-linking to their mRNA targets. Mispairing or partial hybridization by such a reagent could lead to irreversible modification of non-targeted messages, while degradation of the

† This paper is dedicated to Dr. Yoshihisa Mizuno on the occasion of his 75th birthday.

modified oligomer could lead to release of the modifying agent and subsequent reaction with mRNA, DNA or other cellular macromolecules. Both types of non-target interactions could potentially lead to cellular toxicity or other undesirable effects in cell culture experiments.

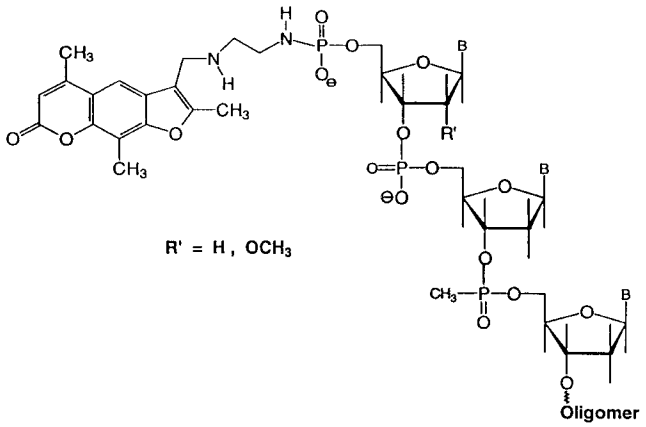
Psoralen-derivatized oligodeoxyribonucleotides effectively cross-link *in vitro* with both single-stranded and double-stranded target nucleic acids when activated by long wavelength ultraviolet light.[1-13] Similarly, oligodeoxyribonucleoside methylphosphonates can be derivatized with psoralen and have also been demonstrated to effectively cross-link with single stranded DNA and messenger RNA *in vitro*.[14-19] The ability of psoralen-derivatized methylphosphonate oligomers to cross-link to messenger RNA suggests that such nucleic acid analogues may be useful in inhibiting gene expression *in vitro* and potentially in cells at the level of mRNA translation.

Previously, we have demonstrated that psoralen-derivatized oligodeoxyribonucleoside methylphosphonates complementary to vesicular stomatitis virus (VSV) N or M messenger RNA are capable of specifically cross-linking with their complementary messages *in vitro*.[20] To further explore the ability of these oligomers to serve as antisense reagents in cells, we have examined their stability in cell culture as well as their ability to inhibit VSV protein synthesis both *in vitro* and in virus infected cells.

RESULTS AND DISCUSSION

Psoralen-Derivatized Oligonucleoside Methylphosphonates Complementary to VSV mRNAs

The general structure of oligonucleoside methylphosphonates derivatized at their 5'-ends with 4'-{[N-aminoethyl)amino]methyl}-4, 5',8-trimethylpsoralen is shown in Figure 1. Three psoralen-derivatized oligonucleoside methylphosphonates, **(ae)AMT-I**, **(ae)AMT-2'-O-me-I** and **(ae)AMT-II** each complementary to either VSV N- or M-mRNA, were synthesized. The sequence of each oligomer is shown at the bottom of Figure 1 and the location of each oligomer binding site on the complementary VSV mRNA is denoted below each oligomer sequence.



d-TpCTGCGCTGGTTCTGG I
VSV N-mRNA 387-402

d-UmpCTGCGCTGGTTCTGG 2'-OMeI
VSV N-mRNA 387-402

d-TpACAGCGGCTGCCACA II
VSV M-mRNA 290-305

FIGURE 1: Structure of a 5'-psoralen-derivatized oligodeoxyribonucleoside methylphosphonate. Shown at the bottom are the sequences of oligomers **I**, **2'-O-me-I** and **II**.

Cell-Free Translation of VSV mRNA Cross-Linked with (ae)AMT-I or (ae)AMT-II *In vitro* cross-linking of **(ae)AMT-I** or **(ae)AMT-II** (5 μM, non-radioactive) with VSV mRNA (2 μg) was carried out at 20°C, as described previously.[20] Following photo-irradiation, the reactions were added to a rabbit reticulocyte lysate translation system which included [^{35}S]-methionine in the reaction for labeling of translated proteins. Only the VSV N-, NS- and M-proteins are synthesized in this system.[21] As a control, VSV mRNA which was preincubated at 20°C in the presence of **(ae)AMT-I** or **(ae)AMT-II** without photo-irradiation was added to a separate translation mixture. Following the translation reactions, the products were electrophoresed on a polyacrylamide/ SDS discontinuous gel, and the resulting autoradiogram of the gels scanned using a laser densitometer to estimate the relative amounts of each protein synthesized in each reaction. *In vitro* translation of VSV mRNA in the presence

of **(ae)AMT-I** following photo-irradiation at 20°C resulted in specific inhibition of VSV N-protein synthesis relative to the synthesis of VSV NS- and M-proteins (Figure 2, panel B). The densitometric peaks for the VSV protein bands were cut from the scans and weighed to obtain the percent inhibition of N-protein synthesis relative to the NS- and M-proteins. Using this method, it was found that under the conditions studied, VSV N-protein synthesis was inhibited 42% relative to M- and NS-protein synthesis. This percent inhibition is consistent with the efficiency of cross-linking of **(ae)AMT-I** to the N message observed at 20°C, as previously determined by both a direct cross-linking method and an AMV reverse transcriptase termination assay.[20]

Similarly, *in vitro* translation of VSV mRNA in the presence of **(ae)AMT-II** following photo-irradiation at 20°C resulted in inhibition of VSV M-protein synthesis by 40% relative to the NS-protein which remained constant. Photo-irradiation of VSV mRNA with **(ae)AMT-II** also resulted in 15% inhibition of VSV N-protein synthesis. This low level of non-specific inhibition could have occurred as a consequence of partial complementarity between **(ae)AMT-II** and the N message, resulting in binding and cross-linking of this oligomer to the N-mRNA and subsequent translational inhibitory effects *in vitro.* However, a computer homology search between the sequence of **(ae)AMT-II** and VSV N-mRNA failed to reveal any significant homology between the two sequences, while cross-reactivity between **(ae)AMT-II** and VSV N message was not observed following cross-linking of the oligomer with VSV mRNA at 20°C.[20] Similar low levels of non-specific inhibition have been observed before in *in vitro* translation experiments using psoralen-derivatized oligonucleoside methylphosphonates targeted to alpha- or beta-globin messenger RNA.[15] The low level of non-specific inhibition may result as a consequence of depleting the available pool of ribosome or other translation factors in the translation mixture, due to ribosomes "piling up" on the targeted, cross-linked message. Such depletion could result in a decrease in the translation of a non-targeted message, particularly if the translational efficiency of the non-targeted message is low relative to the targeted, cross-linked message.

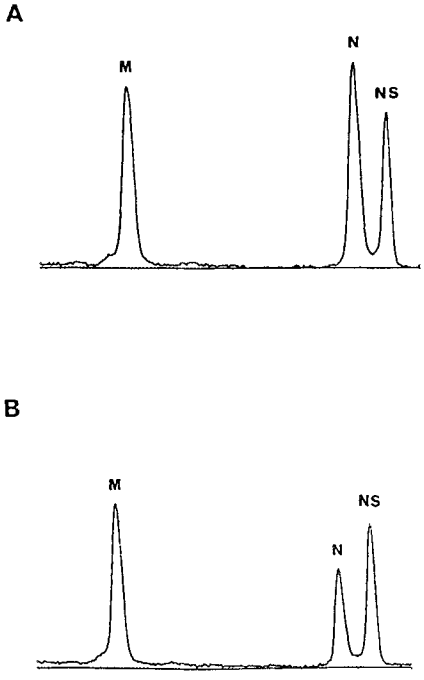


FIGURE 2: Effect of 5 μM **(ae)AMT-I** on VSV protein synthesis in cell-free translation reaction without pre-irradiation (A) or with pre-irradiation (B) of VSV mRNA-oligomer solutions at 20°C. The densitometric scans show the three VSV proteins synthesized in this system, M, N and NS.

Stability of (ae)AMT-I and (ae)AMT-2'-O-me-I in Mouse L-cells

Mouse L- cells were incubated with [^{32}P]-labeled **(ae)AMT-I** or **(ae)AMT-2'-O-me-I** at 37°C. The culture media was removed from the cells after 1, 3, 6, 9, 12 and 24 hrs and the cells were lysed at each time point. Increasing amounts of radioactivity in the lysate of cells incubated with either **(ae)AMT-I** or **(ae)AMT-2'-O-me-I** was observed as the incubation time increased, suggesting that radioactivity from the oligomers was being taken up by the cells (data not shown). Aliquots of the lysate each containing 2,000 dpm of radioactivity were analyzed by polyacrylamide gel electrophoresis. Autoradiograms of these gels are shown in Figure 3B and 3D. For both **(ae)AMT-I** and **(ae)AMT-2'-O-me-I**, intact oligomer was observed in the lysate of cells incubated with each oligomer

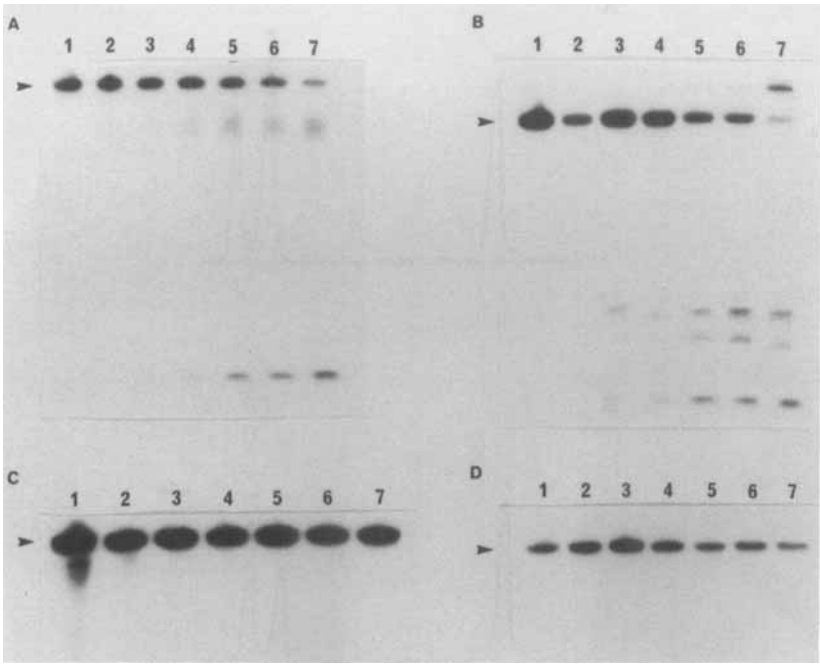


Figure 3: Products in the culture media (A, C) and cell lysate (B, D) after incubation of **(ae)AMT-I** (A, B) or **(ae)AMT-2'-O-me-I** (C, D) with mouse L-cells at 37°C for 1 (lane 2), 3 (lane 3), 6 (lane 4), 9 (lane 5), 12 (lane 6) and 24 (lane 7) hr. Lane 1 in each panel contains untreated oligomer, the position of which is indicated by the arrow.

throughout the 24 hr period. By 24 hrs, most of the radioactivity present in the lysate of **(ae)AMT-I** migrates as shorter material or remains in the well of the gel. This latter material is most likely composed of cellular RNA and/or DNA which has incorporated [^{32}P]-labeled nucleotide resulting from breakdown of the oligomer. These results indicate that **(ae)AMT-I** is not completely resistant to a nuclease activity present in mouse L-cells.

Examination of the structure of **(ae)AMT-I** indicates that a likely point of nuclease attack on the psoralen-derivatized oligomer would be the single, 5'-phosphodiester internucleotide linkage, which could be susceptible to endonucleolytic cleavage. Sproat *et al.* have demonstrated that replacement of the 2'-hydroxyl group on oligoribonucleotides with a 2'-O-methyl group greatly

enhances the stability of these oligomers upon incubation with either RNA or DNA specific nucleases.[22] The 2'-hydrogen on the 5'-nucleotide of **(ae)AMT-I** was replaced with a 2'-O-methyl group by substitution of 2'-O-methyl uridine for thymidine, to give **(ae)AMT-2'-O-me-I**. Upon incubation of **(ae)AMT-2'-O-me-I** with mouse L-cells, only intact oligomer was found in both the lysate and the medium over the 24 hr incubation period (Figure 3D). These results indicate that the 5'-phosphodiester linkage of **(ae)AMT-I** most likely serves as the initial point of endonucleolytic attack and that replacement of the 5'-thymidine of **(ae)AMT-I** with 2'-O-methyl uridine results in stabilization of this linkage with no further breakdown of the oligomer.

Equal volumes of media removed from the cells were also analyzed and the autoradiograms of the gels are shown in Figure 3A and 3C. Intact **(ae)AMT-I** and **(ae)AMT-2'-O-me-I** were both present in the media throughout the 24 hr incubation period. By six hours, the intensity of the **(ae)AMT-I** band begins to decrease, giving rise to two faster migrating species, which increase in intensity over time. The slowest of these two species, running just below **(ae)AMT-I**, comigrates with d-(ae)AMTpT and appears to be actively excluded from the cells. This compound was prepared independently by carbodiimide-mediated coupling of (ae)AMT with thymidine-5'-phosphate. In addition to d-(ae)AMTpT, material which comigrates with inorganic phosphate was also observed running near the bottom of the gel. In contrast to **(ae)AMT-I**, **(ae)AMT-2'-O-me-I** appears completely intact in media incubated with cells over the 24 hr period (Figure 3C).

Effects of (ae)AMT-I and (ae)AMT-II on VSV Protein Synthesis in Cultured Cells Confluent monolayers of mouse L-cells were incubated in media containing 0 μM or 10 μM **(ae)AMT-I** or **(ae)AMT-II** for 20 hrs at 37°C. The medium was then replaced with fresh medium containing 0 μM or 10 μM oligomer. The cells were then infected with VSV and after 6 hrs incubation at 37°C, the infected cells were incubated at 4°C or 20°C for 5 min. Following this incubation period, the cells were irradiated with 365 nm ultraviolet light at 4°C or 20°C for 5 min or 10 min, or kept in the dark at the same temperature. The cells were labeled with [^{35}S]-methionine for 30 min, after which the cells were lysed and the [^{35}S]-labeled proteins analyzed on SDS/polyacrylamide gels.

Treatment of VSV-infected cells with **(ae)AMT-I** resulted in no observable effects on VSV protein synthesis, either in the absence of UV irradiation or following irradiation for 5 min at 4°C or 20°C (data not shown). The lack of inhibitory effects exhibited by this oligomer in the absence of UV irradiation is not unexpected at the concentration of oligomer used (10 μM). Underivatized oligonucleoside methylphosphonates exhibit significant inhibitory effects on protein synthesis in cells only at oligomer concentrations above 75-100 μM.[21] In the absence of UV irradiation, the psoralen-derivatized methylphosphonate oligomers most likely behave in a manner analogous to that of the underivatized oligomers. Therefore, treatment of VSV-infected cells with 10 μM **(ae)AMT-I** would not be expected to result in a significant inhibitory effect on VSV protein synthesis in the absence of UV irradiation. In addition, the lack of any inhibitory effects of **(ae)AMT-I** on VSV protein synthesis in unirradiated, VSV-infected cells is consistent with the observation that *in vitro* translation of N-mRNA is not inhibited by the presence of 5 μM **(ae)AMT-I**.

The lack of VSV protein synthesis inhibition following irradiation of **(ae)AMT-I**-treated cells is surprising given the ability of the oligomer to cross-link to the N message and to inhibit N-protein synthesis *in vitro*. These results suggest that either (1) the oligomer does not reach its target inside the cells, or (2) the oligomer is unable to bind/cross-link to the N message due to the conformation of the oligomer binding site on the N-mRNA inside the cells. With respect to the ability of the oligomer to reach its mRNA target inside the cell, it has previously been shown in our laboratory that the cellular distribution of rhodamine-labeled methylphosphonate oligomers in mouse L-cells reveals a vesicular, punctate pattern of fluorescence, indicative of endosomal compartmentalization of the oligomers.[23] Such endocytotic entrapment of the oligomer may reduce the availability of the internalized oligomer to bind to its target in the cell and exhibit significant antisense effects. With respect to the ability of the oligomer to bind to its target mRNA in the cell, the initial *in vitro* cross-linking experiments between **(ae)AMT-I** and VSV mRNA were carried out under low stringency conditions with little salt, conditions under which the mRNA would be expected to have reduced secondary structure.[20] In contrast,

cellular messenger RNA would be expected to have a significant degree of secondary structure due to the ionic nature of the intracellular environment. In addition, RNA binding proteins may block hybridization of the oligomer to its binding site on the target mRNA in the cell. The lack of VSV protein synthesis inhibition in **(ae)AMT-I**-treated, UV irradiated cells demonstrates that the presence of the psoralen-derivatized oligomer does not exert any non-specific inhibitory effects in UV irradiate cells. Although 10 min UV irradiation of VSV-infected cells did result in a moderate level of non-specific inhibition on protein synthesis (data not shown), this non-specific inhibitory effect was apparent in both untreated and oligomer-treated cells.

Similar to **(ae)AMT-I**, **(ae)AMT-II** had no effect on VSV protein synthesis in unirradiated cells. Once again, **(ae)AMT-II** is presumably behaving in a manner analogous to that of the underivatized methylphosphonate oligomers, where significant inhibitory effects on protein synthesis in cells have only been observed at concentrations above 75-100 μM. However, as shown in Figure 4, irradiation of **(ae)AMT-II**-treated cells for 10 min at 4°C resulted in approximately 70% inhibition of VSV N- and M-proteins, and greater than 90% inhibition of VSV G- and NS-proteins compared to untreated, UV irradiated cells. The inhibitory effects of **(ae)AMT-II** and UV irradiation on VSV protein synthesis appeared to be temperature dependent, as irradiation of **(ae)AMT-II** treated cells at 20°C resulted in no inhibitory effects on protein synthesis upon comparison with untreated, UV irradiated cells (data not shown).

The lack of protein synthesis inhibition upon irradiation of **(ae)AMT-II**-treated cells at 20°C suggests that this oligomer does not cross-link to the mRNA at this temperature, in VSV-infected cells. These results are in contrast to the behavior of **(ae)AMT-II** *in vitro*, where rather extensive and specific cross-linking to the M message was observed at 20°C.[20] It seems likely that at 20°C, the ribosomes are actively translating VSV mRNA in VSV-infected cells. Under these conditions, the oligomer binding site on the M-mRNA may not be accessible to the oligomer. Conversely, any bound oligomer may simply be displaced by translocating ribosomes prior to UV irradiation and cross-linking.

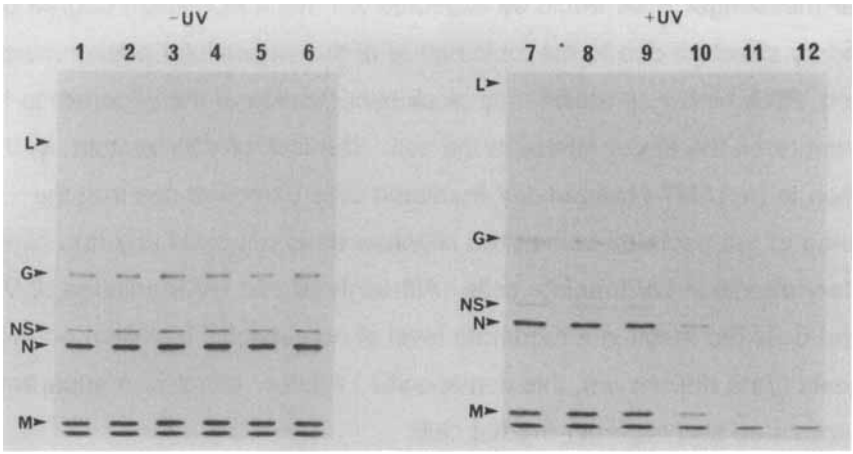


FIGURE 4: [^{35}S]-Labeled proteins isolated from untreated (lanes 1-3) and **(ae)AMT-II**-treated (lanes 4-6), VSV-infected mouse L-cells in the absence of UV irradiation (lanes 1-6), or from untreated (lanes 7-9) and **(ae)AMT-II**-treated (lanes 10-12), VSV-infected mouse L-cells following UV irradiation for 10 min at 4°C (lanes 10-12). The mobilities of the different VSV proteins are shown on the left side of the autoradiogram of the polyacrylamide gels.

Previous studies on the effects of underivatized methylphosphonate oligomers on the translation of rabbit globin mRNA *in vitro* demonstrated that oligomers complementary to the initiation codon region of globin mRNA were more effective inhibitors of translation than those complementary to the coding region of the globin message.[24] These results indicate that, in some instances, ribosomes actively translating mRNA may be capable of displacing oligomers bound to sites within the coding region of the message during translation. However, oligonucleoside methylphosphonates and psoralen-derivatized oligonucleoside methylphosphonates complementary to a site in the coding region of Ha-*ras* mRNA were shown to be effective inhibitors of *ras* p21 expression in c-Ha-*ras*-transformed mouse NIH 3T3 cells.[25] The ability of the cellular translational machinery to remove bound oligomers from the target mRNA may be dependent upon such factors as location of oligomer binding site within the message, RNA secondary structure and the rate at which the message is translated. In contrast to cellular translation at 20°C, placing the

cells at 4°C should reduce or prevent translational activity, and consequently, the mRNA may be accessible to cross-linking by the oligomer. This situation is similar to that of the *in vitro* translation experiments in which the oligomer is first hybridized and cross-linked to the naked mRNA, and translation of the cross-linked message is subsequently initiated by addition of the reticulocyte lysate.

The lack of specific inhibition of M-protein synthesis by **(ae)AMT-II** at low temperature could be due to non-specific cross-linking by the oligomer to the other VSV mRNAs in the cell. However, this seems unlikely because although some cross-reactivity was observed between **(ae)AMT-II** and non-targeted VSV messages at 0°C *in vitro*, the extent of cross-reactivity appeared to be at least 10-fold less than the extent of cross-linking observed with the M message.[20] Thus if non-specific cross-linking alone were responsible for non-specific inhibition of VSV protein synthesis, the M-protein should still be inhibited to a greater extent than the other VSV proteins.

Non-specific inhibition of VSV protein synthesis following UV irradiation of **(ae)AMT-II**-treated, VSV-infected cells at low temperature may be a consequence of cross-linking of **(ae)AMT-II** to its complementary site on the M message, which is located well into the coding region of the M-mRNA. If such cross-linking inhibits the elongation step of translation, ribosomes may "pile up" on the message. This phenomenon could effectively deplete the available pool of active ribosomes and/or other translation factors required for protein synthesis and thus lead to an apparent, overall inhibition of protein synthesis in the cell. Such depletion may be particularly serious in a system such as VSV where one might expect the supply of ribosomes to be limited relative to the amount of mRNA to be translated. If this is the case, then oligomers which cross-link near the initiation codon and consequently prevent initiation of protein synthesis might be expected to exhibit greater specificity in their inhibitory effects on translation. Consistent with this hypothesis is the observation that a psoralen-derivatized methylphosphonate oligomer (12-mer) complementary to nucleotides 50 to 61 of VSV M-mRNA inhibited VSV protein synthesis in VSV-infected cells upon irradiation of the cells at 20°C.[17] The binding site for this oligomer is four nucleotides downstream from the initiation codon of the M

message. M-protein synthesis was inhibited 37% with this oligomer, with the inhibition of NS- and N-protein synthesis occurring to a lesser extent (9% and 14%, respectively).

CONCLUSION

The results of this study demonstrate that derivatization of oligodeoxyribonucleoside methylphosphonates with psoralen creates an oligomer which can cross-link with a target messenger RNA in a sequence-specific manner *in vitro*, is taken up intact by cells and is capable of inhibiting VSV protein synthesis in cell culture. Such antisense reagents should be useful in elucidating the mechanism(s) by which antisense oligonucleotides inhibit gene expression in cells.

EXPERIMENTAL SECTION

Materials Gamma-[^{32}P]-ATP, alpha-[^{32}P]-dCTP and L-[^{35}S]-methionine were purchased from Amersham, Inc. T4 polynucleotide kinase was obtained from United States Biochemicals. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (CDI) was obtained from Sigma Chemicals. Reagents for the preparation of oligodeoxyribonucleoside methylphosphonates were obtained from Cruachem and JBL Scientific, Inc. SEP PAK C-18 reversed phase cartridges were obtained from Waters Associates. The RiboSep mRNA isolation kit was purchased from Collaborative Research, Inc., and the rabbit reticulocyte lysate kit was obtained from Gibco BRL. EN^{3}HANCE solution for treatment of [^{35}S]-protein gels was purchased from ICN Biomedicals, Inc. Gel electrophoresis of oligodeoxyribonucleoside methylphosphonates was carried out on 20 cm x 20 cm x 0.75 mm gels which contained 15% polyacrylamide, 0.089 M Tris, 0.089 M boric acid, 0.2 mM ethylenediamine tetraacetate (1X TBE) and 7 M urea. Samples were dissolved in loading buffer which contained 90% formamide, 0.089 M Tris, 0.089 M boric acid, 0.2 mM ethylenediamine tetraacetate, 0.2% bromophenol blue and 0.2% xylene cyanol. Solutions containing RNA were prepared using water which had been previously treated with diethylpyrocarbonate. Products of the cell-free translation of VSV mRNA and

[^{35}S]-labeled proteins isolated from VSV-infected cells were analyzed on 20 cm x 20 cm x 0.15 cm polyacrylamide/SDS discontinuous gels which consisted of a 5% polyacrylamide stacking gel and a 10% polyacrylamide resolving gel. The loading buffer for the discontinuous gels contained 0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol and 10% mercaptoethanol. The running buffer for the discontinuous gels contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS, buffered at pH 8.3.

Preparation of Psoralen-Derivatized Oligodeoxyribonucleoside Methylphosphonates Oligodeoxyribonucleoside methylphosphonates were synthesized on controlled pore glass supports using 5'-O-(dimethoxytrityl)-deoxyribonucleoside 3'-O-methyl-N,N-diisopropylphosphonamidite synthons using a Biosearch 8700 DNA synthesizer.[26] The oligomers were deprotected and purified as previously described.[26, 27]

Psoralen-derivatized oligonucleoside methylphosphonates were prepared as previously described.[28] Oligomers **(ae)AMT-I** and **(ae)AMT-II** were prepared by incubating 666 nmoles, 100 A_{254} units of oligomer **I** or **II** in 836 µL of a solution containing 5% acetonitrile, 5 mM dithiothreitol, 50 mM Tris, pH 7.6, 5 mM magnesium chloride, 4.8 mM ATP and 900 units of polynucleotide kinase. The solution was incubated for 14 hrs at 37°C. The solution was evaporated to dryness; the residue was dissolved in 875 µL of a 0.1 M imidazole buffer, pH 6.0; and the solution was treated with 150 µL of acetonitrile and 100 µL of 1 M CDI at room temperature for 4 hrs. After evaporation, the residue was first extracted three times with 0.6 mL of methanol/triethylamine (1:0.0125, v/v) to remove excess imidazole and CDI and then evaporated to remove residual methanol/triethylamine. The residue was then dissolved in 700 µL of 0.25 M lutidine hydrochloride buffer, pH 7.5 and incubated with a solution containing 165 µL of acetonitrile and 175 µL of 0.05 M 4'-{[N-(aminoethyl)-amino]methyl}-4,5',8-trimethylpsoralen, (ae)AMT, for 48 hrs at room temperature. Solvents were removed by evaporation and the residue extracted three times with 0.6 mL of methanol/triethylamine (1:0.0125, v/v) to remove excess (ae)AMT. The product was purified by preparative HPLC on a Microsorb C-18 reversed-phase

column and desalted on a SEP PAK C-18 reversed-phase cartridge. The final product was obtained in 20% overall yield.

[^{32}P]-Labeled, psoralen-derivatized oligonucleoside methylphosphonates [**(ae)AMT-I** and **(ae)AMT-2'-O-me-I**] were prepared in a similar manner. Three nmoles, 0.42 A_{254} units, of **I** or **2'-O-me-I** were dissolved in 15 μL of a solution containing 5 mM dithiothreitol, 50 mM Tris, pH 7.6, 5 mM magnesium chloride, 0.13 mM ATP, 30 μCi gamma-[^{32}P]-ATP and 30 units of polynucleotide kinase and the solution was incubated at 37°C for 4 hrs. The solution was evaporated to dryness; the residue was dissolved in 72 μL of a 0.1 M imidazole buffer, pH 6.0; and the solution was treated with 8 μL of 1 M CDI at room temperature for 4 hrs. The reaction mixture was evaporated to dryness and the residue was extracted three times with 0.2 mL of methanol/triethylamine (1:0.0125, v/v) to remove excess imidazole and CDI. The residue was evaporated to remove residual methanol/triethylamine and dissolved in 70 μL of 0.25 M lutidine hydrochloride buffer, pH 7.5. A solution containing 20 μL of acetonitrile and 30 μL of 0.05 M (ae)AMT was added and the reaction was incubated for 16 hrs at room temperature. The reaction mixture was then evaporated to dryness and the residue was extracted three times with 0.2 mL of methanol/triethylamine (1:0.0125, v/v) to remove excess (ae)AMT. After evaporation, the residue was dissolved in gel loading buffer and electrophoresed on a 15% polyacrylamide gel. Bands corresponding to (ae)AMT-[^{32}P]-oligonucleoside methylphosphenates were located by autoradiography, cut from the gel, and each oligomer was extracted from the gel with four 0.5 mL portions of 1 M triethylammonium bicarbonate, pH 7.5. The combined extracts were diluted with 8 mL of 0.1 M sodium phosphate, ph 5.8, and desalted on a SEP PAK C-18 reversed-phase cartridge.[28] The psoralen-derivatized oligomers were obtained in approximately 25% overall yield.

Cell-Free Translation Assay of VSV mRNA Cross-Linked with (ae)AMT-I or (ae)AMT-II *In vitro* photo-cross-linking of **(ae)AMT-I** or **(ae)AMT-II** (5 μM, non-radioactive) with VSV mRNA (4 μg) was carried out at 20°C, as previously described.[20] Following photo-irradiation, the solutions were diluted with 30 μL of water. Ten μL of this solution was then aliquoted into

three siliconized, borosilicate test tubes (10 mm x 75 mm). To each tube was added 3 µL of a solution containing 250 mM HEPES, pH 7.2, 400 mM potassium chloride, 100 mM creatine phosphate, 500 µg/mL calf liver tRNA, 19 amino acids (500 µM each), 2.4 µL of a 1 M potassium acetate solution, pH 7.2, 1.05 µL of 20 mM magnesium acetate, pH 7.2, 1.65 µL of [^{35}S]-methionine (specific activity > 800 Ci/mol) and 10 µL of rabbit reticulocyte lysate. The translation reactions were incubated at 30°C for one hour. Three µL aliquots were removed from each reaction and added to 10 µL of 2X gel loading buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 2% bromophenol blue). The samples were vortexed briefly, placed in a boiling water bath for 2 min and electrophoresed on polyacrylamide/SDS discontinuous gels consisting of a 5% polyacrylamide stacking gel and a 10% polyacrylamide resolving gel. Electrophoresis was carried out using a buffer consisting of 0.025 M Tris, 0.192 M glycine and 0.1% SDS, buffered at pH 8.3. Gels were fixed overnight in 50% methanol/10% acetic acid at room temperature, soaked in EN^{3}HANCE solution (NEN-Dupont) for one hour and in water for thirty minutes, and subsequently dried onto Whatman 3MM paper under vacuum. Dried gels were autographed at -80°C and the autoradiograms subsequently scanned using an LKB Ultrascan densitometer to measure the levels of VSV protein synthesis.

Incubation of (ae)AMT-I or (ae)AMT-2'-O-me-I with Mouse L-Cells

Mouse L929 fibroblasts were grown until confluent in 2 cm, 24 well plates. The medium was replaced with 0.4 mL of 1X EMEM/10% fetal calf serum containing either 1 x 10^6 dpm of 80 nM [^{32}P]-labeled **(ae)AMT-I** or 1 x 10^6 of 80 nM [^{32}P]-labeled **(ae)AMT-2'-O-me-I**. Medium was removed after 1, 3, 6, 9, 12 or 24 hrs and stored at -20°C for later analysis. The cells were washed once with 0.25 mL of ice-cold calcium-magnesium free phosphate buffered saline and then lysed with 0.1 mL of 0.5% NP-40 for 5 min at 0°C. Aliquots containing 2 µL of medium or aliquots containing 2,000 dpm of lysate were evaporated to dryness; the residue was dissolved in 10 µL of gel loading buffer and the samples electrophoresed for 30 min at 1,000 volts on a 20% polyacrylamide gel containing 7 M urea.

Effects of Psoralen-Derivatized Oligodeoxyribonucleoside Methylphosphonates on VSV Protein Synthesis in Virus-Infected Cells

Monolayers of L929 cells were grown in LAB-TEK 1 cm^2, 8-chamber microscope slides. Wells containing confluent cells at a density of 1.0-1.2 x 10^5 cells/well were preincubated at 37°C with 10 μM **(ae)AMT-I** or **(ae)AMT-II** in 1X EMEM/2% fetal calf serum or with oligomer-free medium for 20 hrs. Following this incubation period, media was removed and replaced with fresh medium containing 10 μM oligomer. Cells were infected with VSV at a multiplicity of infection of five plaque forming units per cell. The VSV-infected cells were incubated for an additional 6 hrs at 37°C. The cells were then incubated at 4°C or 20°C for 5 min after which they were irradiated at 365 nm for either 5 or 10 min at 4°C or 20°C. Control wells containing oligomer or no oligomer were kept in the dark at 4°C or 20°C for 5 or 10 min. Following irradiation, the media was removed and the cells were washed with 0.2 mL of ice-cold DPBS. The DPBS was then replaced with 0.1 mL of methionine-free medium containing 83 μCi/mL [^{35}S]-methionine. The cells were incubated for 30 min at 37°C after which the cells were washed once with 0.2 mL of a buffer containing 10 mM Tris, pH 7.4, 2.5 mM magnesium chloride and 30 mM potassium chloride (buffer A). Buffer A was removed and replaced with 0.1 mL of buffer A supplemented with 0.75% NP-40. Following incubation on ice for 5 min, cells from each chamber were scraped with a pipet tip, and 30 μL of lysate were removed and transferred to 1.4 mL eppendorf tubes which contained 30 μL discontinuous gel loading buffer consisting of 0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol and 10% mercaptoethanol. Each sample was placed in a boiling water bath for 2 min prior to loading onto the gel. Fifteen μL aliquots were loaded onto 20 cm x 20 cm x 0.15 cm discontinuous polyacrylamide/SDS gels and electrophoresed as described for the cell-free translation assay. Gels were fixed overnight, treated with EN^{3}HANCE solution and dried onto Whatmann 3MM paper. Dried gels were autoradiographed at -80°C and the autoradiograms scanned using an LKB ultrascan densitometer to measure the levels of VSV protein synthesis.

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FOOTNOTES

1. Abbreviations used: VSV - vesicular stomatitis virus; **(ae)AMT-I** - oligonucleoside methylphosphonate oligomer #1 derivatized with 4'-{[N-(aminoethyl)amino]methyl}-4,5',8-trimethylpsoralen; (ae)AMT - 4'-{[N-(aminoethyl)amino]methyl}-4,5',8-trimethylpsoralen; HPLC - high performance liquid chromatography; EMEM - Eagle's minimal essential medium with Earle's salts; HEPES - N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid; DPBS - Dulbecco's phosphate buffered saline; SDS - sodium dodecyl sulfate

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